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(12) **United States Patent
Burden**(10) **Patent No.: US 9,329,182 B2**
(45) **Date of Patent: May 3, 2016**(54) **METHOD OF TREATING MOTOR NEURON
DISEASE WITH AN ANTIBODY THAT
AGONIZES MUSK**7,816,322 B2 10/2010 Fallon et al.
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U.S.C. 154(b) by 0 days.(21) Appl. No.: **14/358,055**(22) PCT Filed: **Nov. 14, 2012**(86) PCT No.: **PCT/US2012/065023**

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28, 2012, provisional application No. 61/559,309,
filed on Nov. 14, 2011.(51) **Int. Cl.****A61K 39/395** (2006.01)**A61P 21/00** (2006.01)**C07K 16/28** (2006.01)**G01N 33/573** (2006.01)**A61K 31/7088** (2006.01)**A61K 38/00** (2006.01)**C07K 14/705** (2006.01)(52) **U.S. Cl.**CPC **G01N 33/573** (2013.01); **A61K 31/7088**
(2013.01); **A61K 38/005** (2013.01); **A61K****39/3955** (2013.01); **A61K 39/39541** (2013.01);**C07K 16/286** (2013.01); **C07K 14/705**(2013.01); **C07K 16/28** (2013.01); **C07K****2317/33** (2013.01); **C07K 2317/75** (2013.01);**G01N 2500/04** (2013.01)(58) **Field of Classification Search**

None

See application file for complete search history.

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Primary Examiner — Zachary Howard(74) *Attorney, Agent, or Firm* — Klauber & Jackson LLC(57) **ABSTRACT**Agents, compositions, and medicaments that pertain to
modulating the activity of muscle specific kinase receptor
(MuSK) and methods and uses thereof to modulate MuSK
activity are encompassed herein. Also encompassed are
screening assays to identify modulators of MuSK activity.
Agents identified using the screening assays described herein
are envisioned for use as therapeutics, alone or in composi-
tions or in medicaments, to alleviate or delay motor dysfunc-
tion in subjects afflicted with a disorder associated with nerve
terminal loss or fragmentation, such as amyotrophic lateral
sclerosis, sarcopenia, or anti-MuSK myasthenia gravis.**8 Claims, 8 Drawing Sheets**

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Figure 1

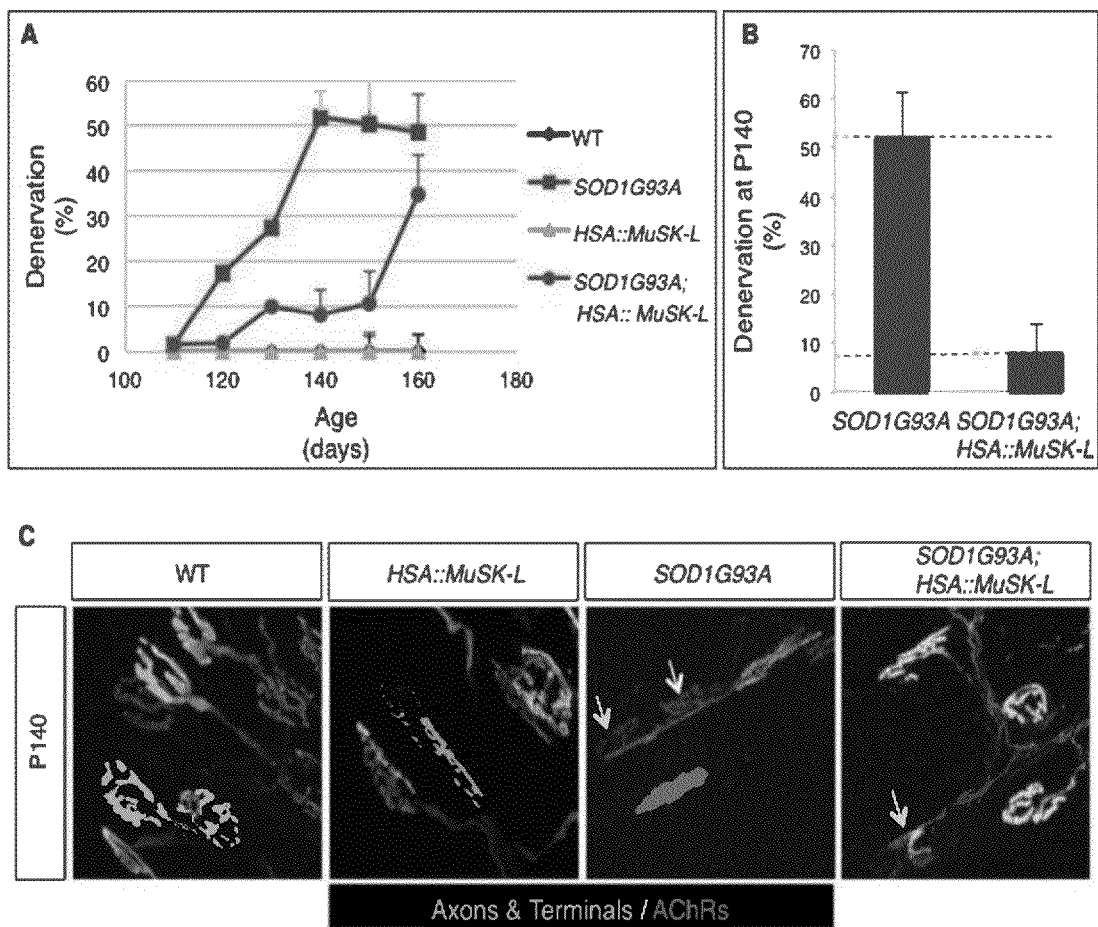


Figure 2

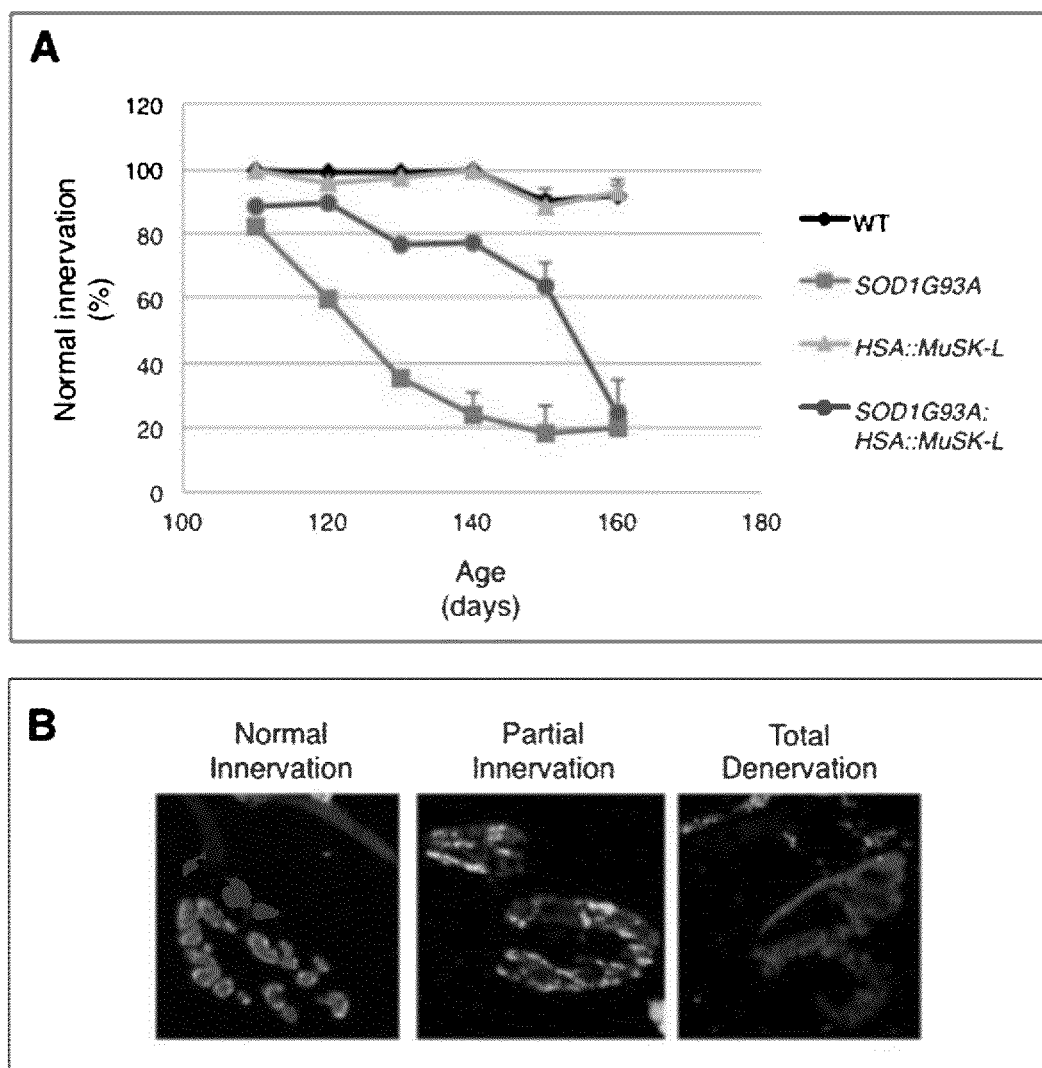


Figure 3

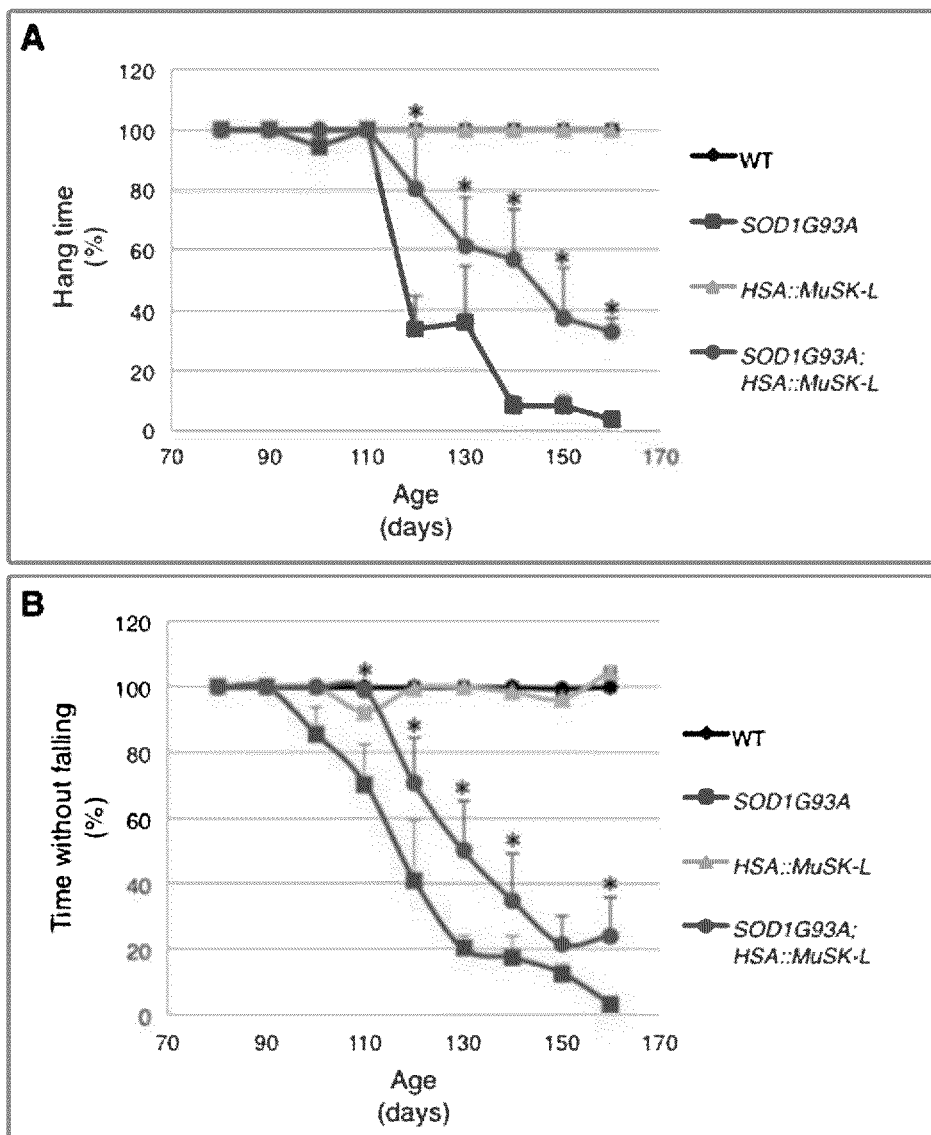


Figure 4

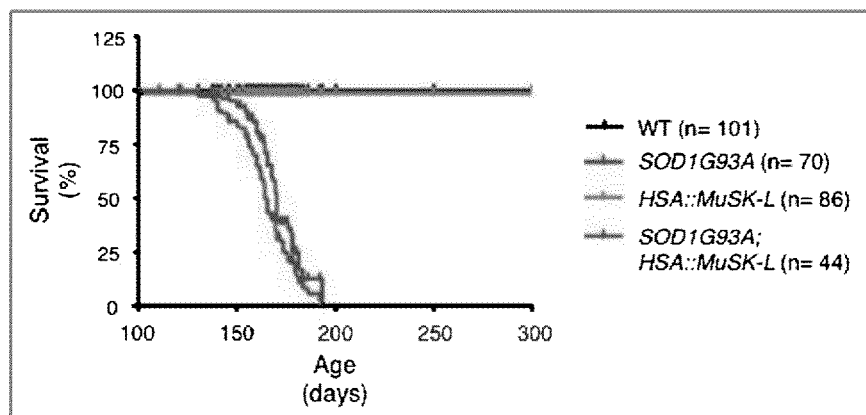
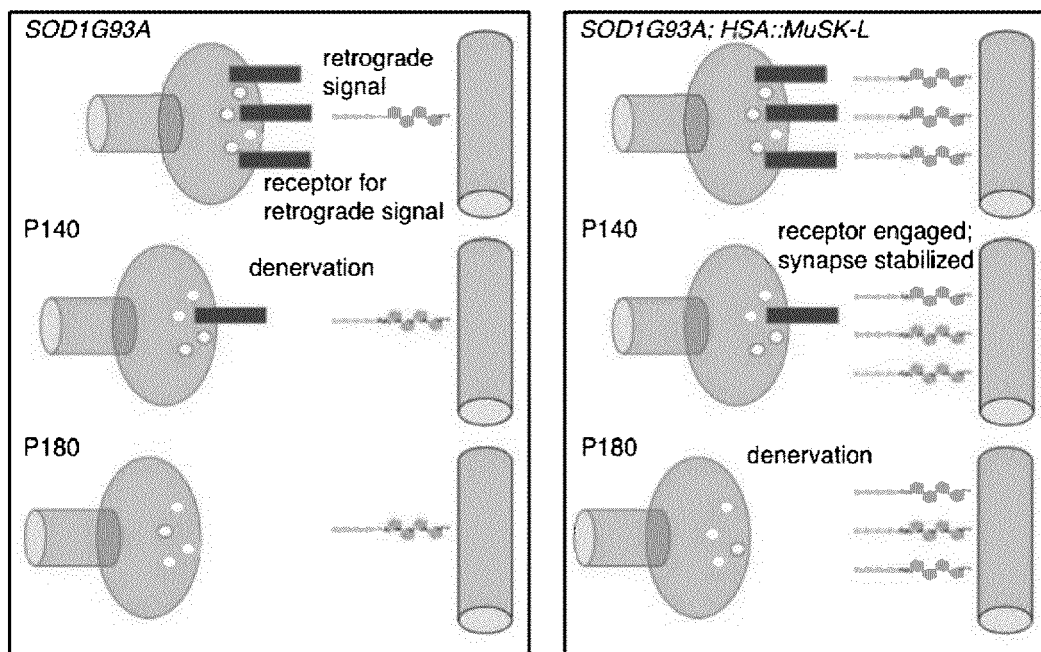
A**B**

Figure 5

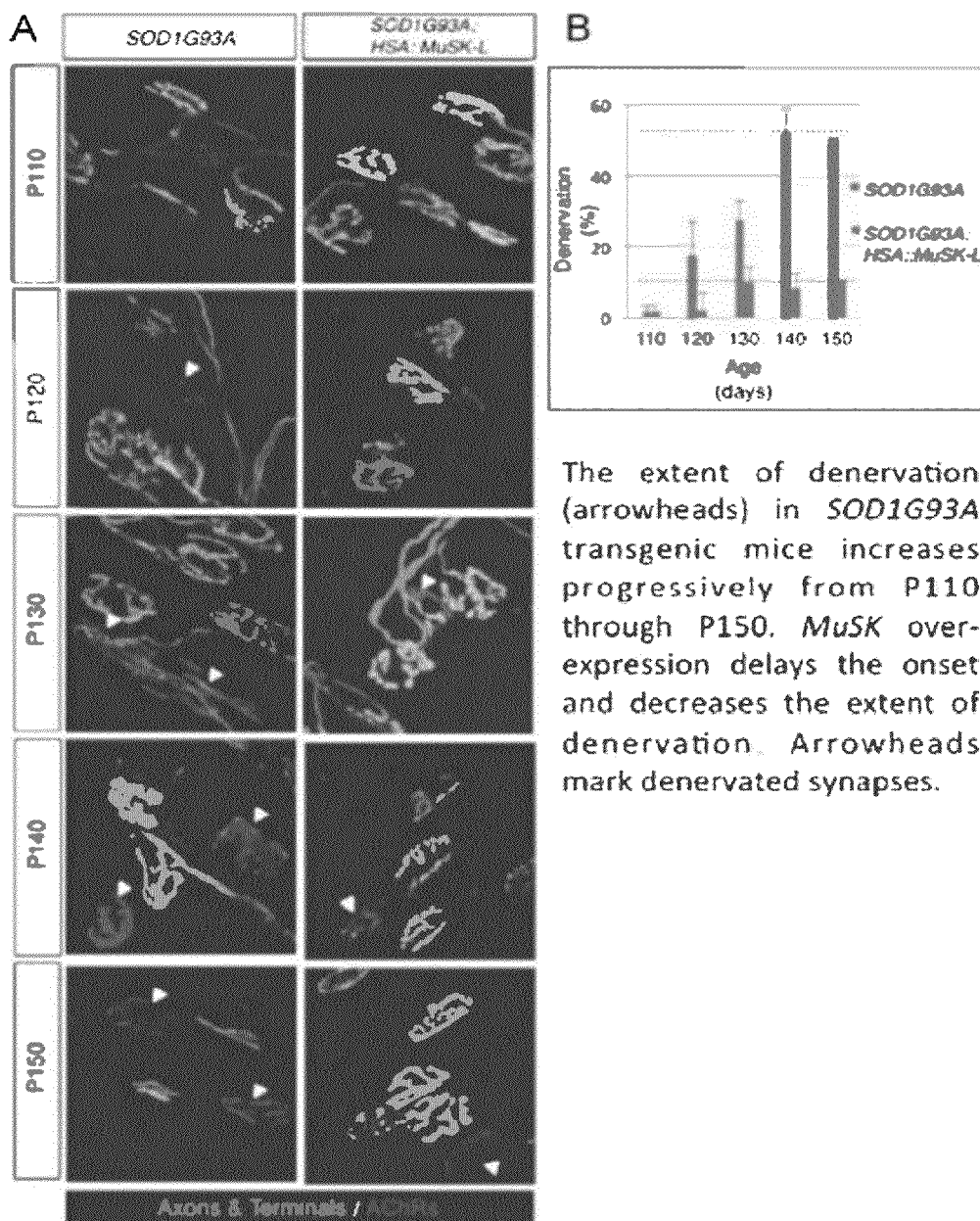
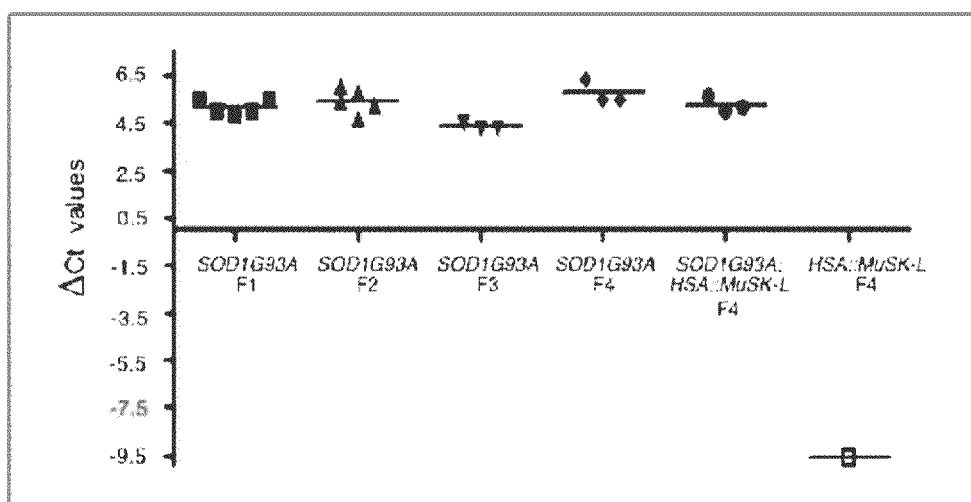


Figure 6



The copy number of the *SOD1G93A* transgene was measured by real time PCR, which showed the the copy number did not change over multiple generations and the course of the experiments.

Figure 7

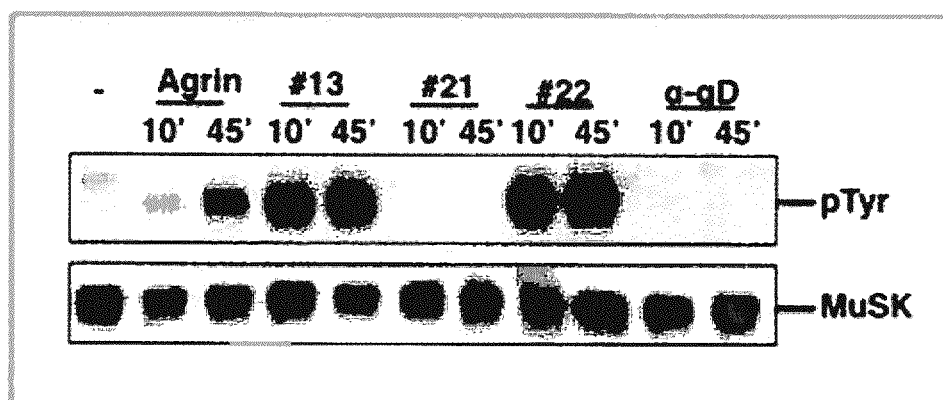


Figure 8

VH

	F1	CDR1	F2
SEQ ID NO: 5	1	MAQVQLQESGGEMKKPGESLKISCKGYGYSFATSWIGWVRQMPGRGLEWM	
SEQ ID NO: 6	1	MAEVQLVQSGGGLVKPGGSLRLSCAASGFTFSDDYMSWIRQAPGKGLEWV	
SEQ ID NO: 7	1	MAEVQLVQSGGGVVQPGGSLRLSCAVSGITLRTYGMHWVRQAPGKGLEWV	
SEQ ID NO: 8	1	MAQVQLVQSGGGLVVRPGGSLRLSCAVSGITLRTYGMHWVRQAPGKGLEWV	
SEQ ID NO: 9	1	MAQVQLVESGGGLVKPGGSLRLSCAASGFTFSHNMNWVRQAPGKGLEWV	
SEQ ID NO: 10	1	MAQVQLQCSGPGLVKPSSETLSLTCTVSGDSISSYYSWIRQPPGKGLEWI	

CDR2

	F3
SEQ ID NO: 5	51 AIMYPGNSDTRHNPSFEDQVTMSADTSINTAYLOWSSLKASDTAMYYCAR
SEQ ID NO: 6	51 SYISSSGSTIIYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAR
SEQ ID NO: 7	51 AGISFDGRSEYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAR
SEQ ID NO: 8	51 AGISFDGRSEYYADSVQGRFTISRDSKNTLYLQMNSLRAEDTAVYYCAR
SEQ ID NO: 9	51 SSISSSSSYIIYYADSVKGRFTISRDN AKNSLYLQMNSLRAEDTAVYYCAR
SEQ ID NO: 10	51 GYIYYSGS--TNYNPSLKSRVTISVDTSKSQFSLKLSVTAADTAVYYCAR

CDR3

	F4	VL	F1
SEQ ID NO: 5	101	AGVAGGAFDLWGKGTMTVTVSSGGGSGGGGSGGGGSQSVLTQ--PASVSGS	
SEQ ID NO: 6	101	-WSGEDAFDIWGQGTMTVTVSSGGGSGGGGSGGGGSDIVMTQSPSTLSAS	
SEQ ID NO: 7	101	-DRGSYGMDVWGRGTMTVTVSSGGGSGGGGSGGGGSDIQMTQSPSTLSAS	
SEQ ID NO: 8	101	-G-AHYGFDIWGQGTMTVTVSSGGGSGGGGSGGGGSDIQMTQSPSTLSAS	
SEQ ID NO: 9	101	-DRGSTGMDVWGRGTLVTVSSGGGSGGGGSGGGGSDIQMTQSPSTLSAS	
SEQ ID NO: 10	100	----GRYFDVWGRGTMTVTVSSGGGSGGGGSGGGGSSYVLTQ--PPSVSGS	

CDR1

	F2	CDR2
SEQ ID NO: 5	150	PGQSITISCTGTSSGVGGYNYVSWYQQHPGKAPKLLIYGNSNRPSGVDPDR
SEQ ID NO: 6	150	VGDRVAITCRASE---GIYHWLAWYQQKPGKAPKLLIYKASSLASGAPSR
SEQ ID NO: 7	150	IGDRVTTTCRASE---GIYHWLAWYQQKPGKAPKLLIYKASSLASGAPSR
SEQ ID NO: 8	149	IGDRVTTTCRASE---GIYHWLAWYQQKPGKAPKLLIYKASSLASGAPSR
SEQ ID NO: 9	150	IGDRVTTTCRASE---GIYHWLAWYQQKPGKAPKLLIYKASSLASGAPSR
SEQ ID NO: 10	145	PGQSITISCTGTSSDVGGYNYVSWYQQHPGKAPKLMYEGSKRPSGVSNR

F3

	CDR3	F4
SEQ ID NO: 5	200	FSASKSGNTASLTISGLQAEDRDYFCSTYAPPGIIMFGGGTKLTVLGAA
SEQ ID NO: 6	197	FSGSGSGADFTLTISLQPDDEFATYYCQQYSNYPL-TFGGGTKLEVKRAA
SEQ ID NO: 7	197	FSGSGSGTDFTLTISLQPDDEFATYYCQQYSNYPL-TFGGGTKLEILRAA
SEQ ID NO: 8	196	FSGSGSGTDFTLTISLQPDDEFATYYCQQYSNYPL-TFGCGTLEIKRAA
SEQ ID NO: 9	197	FSGSGSGTDFTXLTISLQPDDEFATYYCQQYSNYPL-TFGCGTKLEIKRAA
SEQ ID NO: 10	195	FSGSKSGNTASLTISGLQAEDRDYFCSSYTRSTRVFGGGTKLTVLGAA

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METHOD OF TREATING MOTOR NEURON DISEASE WITH AN ANTIBODY THAT AGONIZES MUSK

CROSS REFERENCE TO RELATED APPLICATIONS

The present application is a National Stage Application which claims priority under 35 U.S.C. §120 from co-pending PCT Application No. PCT/US2012/065023 filed Nov. 14, 2012, which in turn claims priority under from 35 U.S.C. §119(e) from U.S. Provisional Application Ser. No. 61/693,857, filed Aug. 28, 2012 and U.S. Provisional Application Ser. No. 61/559,309, filed Nov. 14, 2011, each of which applications are herein specifically incorporated by reference in their entireties.

FIELD OF INVENTION

The compositions and methods described herein relate to muscle specific kinase receptor (MuSK) and modulation thereof, as well as to screening assays to identify modulators of MuSK activity. Agents identified using the screening assays described herein are envisioned for use as therapeutics to alleviate or delay motor dysfunction in subjects afflicted with a disorder associated with nerve terminal loss or fragmentation, including sarcopenia, amyotrophic lateral sclerosis and anti-MuSK myasthenia gravis.

BACKGROUND OF INVENTION

Our ability to move and breathe depends upon the neuromuscular synapse. As such, the neuromuscular synapse is the single synapse essential for survival. Neuromuscular synapse formation is a multi-step process requiring coordinated interactions between motor neurons and muscle fibers, which eventually lead to the formation of a highly specialized postsynaptic membrane and a highly differentiated nerve terminal. As a consequence, acetylcholine receptors (AChRs) become highly concentrated in the postsynaptic membrane and arranged in perfect register with active zones in the presynaptic nerve terminal, and thus ensure fast, robust and reliable synaptic transmission. The signals and mechanisms responsible for this process are poorly understood but require MuSK, a receptor tyrosine kinase that is expressed in skeletal muscle, Agrin, a motor neuron-derived ligand that stimulates MuSK phosphorylation, and Lrp4, the receptor for Agrin. These genes play critical roles in synaptic differentiation, as synapses do not form in their absence, and mutations in MuSK or downstream effectors lead to a reduced number of AChRs at synapses and are a major cause of a group of neuromuscular disorders, termed congenital myasthenia. Moreover, auto-antibodies to AChRs, MuSK or Lrp4, which cause accelerated degradation of AChRs and structural disorganization of the synapse, are responsible for myasthenia gravis.

The mechanisms that control and stabilize connections between motor axon terminals and muscle fibers are poorly understood, but the maintenance of neuromuscular synapses is essential to sustain synaptic transmission and prevent muscle atrophy. In amyotrophic lateral sclerosis (ALS), a loss of motor axon terminals, which causes muscle denervation, is the earliest known sign of disease. ALS is a devastating, neurodegenerative disorder, with a midlife onset, characterized by the progressive loss of upper and lower motor neurons and leading to relentless, lethal paralysis. Following diagnosis of ALS, either sporadic or familial, the time course of

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disease progression varies in an unpredictable manner. Dominant mutations in superoxide dismutase (SOD1) are responsible for 2% of the cases of ALS. Transgenic mice expressing mutant forms of SOD1 develop a paralytic motor neuron disease, which exhibits all of the hallmark features of ALS.

A progressive and gradual withdrawal of motor axon terminals accompanies aging, compromising synaptic function and causing muscle atrophy, or sarcopenia. Once this cycle is initiated, muscle atrophy and deterioration are further exacerbated, as muscle atrophy and a loss of muscle strength reduce physical activity and exercise, which decreases synaptic activity still further. Sarcopenia is a major and debilitating consequence of aging. Moreover, because sarcopenia reduces physical activity, sarcopenia compromises health by contributing to a wide range of complications in multiple organ systems.

The citation of references herein shall not be construed as an admission that such is prior art to the present invention.

SUMMARY OF THE INVENTION

The present inventor sought to determine whether increasing MuSK activity might be sufficient to stabilize synapses that are weakened, irrespective of the mechanism underlying the instability. To this end, the present inventor investigated whether MuSK over-expression might stabilize synapses in mice carrying dominant mutations in SOD1. Mice carrying transgenes with a mutation in SOD1, namely SOD1G93A, serve as a model for ALS, as the pathological and clinical signs of disease in this mouse model replicate those in ALS. In particular, a loss of neuromuscular synapses and the withdrawal of motor axons is the earliest sign of disease in the mouse model and in familial and sporadic forms of ALS. Such muscle denervation leads to paralysis, respiratory failure and ultimately premature death.

The present inventor crossed MuSK-L mice with SOD1G93A mice, which generated four types of progeny: (1) wild-type mice; (2) MuSK-L; (3) SOD1G93A; and (4) MuSK-L; SOD1G93A mice. Although MuSK over-expression did not significantly prolong the longevity of SOD1G93A mice, MuSK over-expression substantially delayed denervation of skeletal muscle in MuSK-L; SOD1G93A mice relative to SOD1G93A mice. Indeed, the loss of neuromuscular synapses was far less in MuSK-L; SOD1G93A mice than SOD1G93A mice during a forty day period (P120-P160) when synapses disassemble in SOD1G93A mice. MuSK over-expression also substantially and similarly improved motor function of SOD1G93A mice, as assessed by an improvement in their ambulatory behavior and an increase in muscle strength during this forty day period when SOD1G93A mice showed severe signs of motor dysfunction and muscle weakness.

The present findings, therefore, demonstrate that increasing MuSK activity in muscle is sufficient to delay synaptic loss and motor dysfunction in a mouse model of ALS. Accordingly, increasing MuSK activity in humans with ALS has the potential to alleviate and delay motor dysfunction, allowing ALS patients to function without assistance for extended periods of time.

Moreover, these findings raise the possibility that increasing MuSK activity may alleviate motor dysfunction in other disorders associated with nerve terminal loss or fragmentation, including sarcopenia. Further, as disease-causing auto-antibodies to MuSK interfere with MuSK function, increasing MuSK activity in patients with anti-MuSK auto-immune myasthenia gravis may likewise alleviate the weakness and fatigue that are hallmark features of this disease.

Accordingly, in an aspect of the invention, a method for delaying motor dysfunction in a subject is presented, the method comprising: administering an agent that increases muscle specific receptor kinase (MuSK) activity to the subject in a therapeutically effective amount sufficient to increase MuSK activity in the subject and thereby improve motor function in the subject. Also encompassed herein is a method for treating a disease or condition associated with synaptic loss and/or motor dysfunction in a subject, the method comprising administering a therapeutically effective amount of an agent that increases MuSK activity to the subject. In another aspect, a method for preserving neuromuscular synapses in a subject is presented, the method comprising: administering an agent that increases muscle specific receptor kinase (MuSK) activity to the subject in a therapeutically effective amount sufficient to increase MuSK activity in the subject and thereby preserve neuromuscular synapses in the subject.

Use of an agent that increases MuSK activity for the treatment of a disease or condition associated with synaptic loss and/or motor dysfunction in a subject comprising administering a therapeutically effective amount of the agent to the subject is also encompassed. An agent that increases MuSK activity for use in treating a disease or condition associated with synaptic loss and/or motor dysfunction in a subject is also encompassed, wherein the agent is administered to the subject in a therapeutically effective amount so as to treat the disease or condition associated with synaptic loss and/or motor dysfunction in the subject. Use of an agent that increases MuSK activity to delay motor dysfunction in a subject comprising administering a therapeutically effective amount of the agent to the subject is also envisioned. An agent that increases MuSK activity for use in delaying motor dysfunction in a subject is further envisioned, wherein the agent is administered to the subject in a therapeutically effective amount to delay motor dysfunction in the subject.

Also encompassed herein is use of an agent that increases MuSK activity for the preparation of a medicament for treating a disease or condition associated with synaptic loss and/or motor dysfunction in a subject or for delaying motor dysfunction in a subject.

In an embodiment of the methods and uses described herein, the agent is a polypeptide, an antibody or antibody fragment, a polynucleotide, a chemical compound, or a small molecule. In a more particular embodiment, the antibody or antibody fragment increases MuSK activity upon binding to MuSK.

In another embodiment of the methods and uses described herein, the agent is a polypeptide comprising or consisting of the amino acid sequence of agrin or Lrp4 or functional fragments thereof. Exemplary amino acid sequences of human agrin are designated herein as SEQ ID NO: 2. Exemplary amino acid sequences of human Lrp4 are designated herein as SEQ ID NO: 4.

In a particular embodiment, the delay in motor dysfunction is measurable by determining innervation levels. In another embodiment, administering the agent maintains innervation levels in the subject.

In yet another embodiment, administering the agent stabilizes motor axon synapses or increases the number of motor axon synapses in the subject.

In a particular embodiment, the subject is suffering from amyotrophic lateral sclerosis, sarcopenia, or myasthenia gravis. In a more particular embodiment, the subject is suffering from amyotrophic lateral sclerosis. More particularly still, the subject may be a mammal (for example, a mouse, rat, or primate). In a more particular example, the mammal is a mouse comprising a transgene with a mutation in SOD1. In an

even more particular embodiment, the mouse comprises a transgene with a mutation in SOD1, wherein the transgene is SOD1G93A. In another particular embodiment, the mammal is a human.

Also encompassed herein is a method for screening to identify an agent that increases MuSK activity, the method comprising: contacting populations of myotubes with at least one agent in vitro and assaying the MuSK activity in the populations after the contacting, wherein an increase in MuSK activity in a population of myotubes following contact with the at least one agent relative to that following contact with a control agent identifies the at least one agent as an agent that increases MuSK activity.

In a particular embodiment of the screening method, the myotubes are differentiated from muscle cells grown in vitro. In a more particular embodiment, the myotubes or muscle cells are isolated from a mouse comprising a transgene with a mutation in SOD1. In an even more particular embodiment, the mouse comprises a transgene with a mutation in SOD1, wherein the transgene is SOD1G93A.

In an embodiment of the screening method, the MuSK activity is measured by detecting MuSK phosphorylation levels and/or clustering of acetylcholine receptors (AChR). More particularly, an increase in MuSK activity may be measured by detecting increased levels of MuSK phosphorylation and/or clustering of acetylcholine receptors (AChR) following contacting with the at least one agent.

In another particular embodiment of the screening method, the at least one agent is a polypeptide, an antibody or antibody fragment, a polynucleotide, a chemical compound, or a small molecule.

In another embodiment of the screening methods described herein, the agent is a polypeptide comprising or consisting of the amino acid sequence of agrin or Lrp4 or functional fragments thereof. Exemplary amino acid sequences of human agrin are designated herein as SEQ ID NO: 2. Exemplary amino acid sequences of human Lrp4 are designated herein as SEQ ID NO: 4.

The screening method may further comprise a secondary assay, wherein the at least one agent identified as an agent that increases MuSK activity in the primary screen is evaluated in a secondary assay wherein MuSK is immunoprecipitated from cell lysates and analyzed by probing Western blots with antibodies to phosphotyrosine.

Also encompassed herein is a method for delaying motor dysfunction in a subject, the method comprising: administering an agent that increases muscle specific receptor kinase (MuSK) activity as identified by a screening method described herein to the subject in a therapeutically effective amount sufficient to increase MuSK activity in the subject and thereby improve motor function in the subject.

In a further embodiment, a method for preserving neuromuscular synapses in a subject is envisioned, the method comprising: administering an agent that increases muscle specific receptor kinase (MuSK) activity as identified by a screening method described herein to the subject in a therapeutically effective amount sufficient to increase MuSK activity in the subject and thereby preserve neuromuscular synapses in the subject.

In a still further embodiment, use of an agent that increases muscle specific receptor kinase (MuSK) activity as identified by a screening method described herein in the preparation of a medicament for delaying motor dysfunction in a subject is envisioned, wherein the medicament is administered in a therapeutically effective amount that increases MuSK activity in the subject and thereby improves motor function in the subject.

In yet another embodiment, use of an agent that increases muscle specific receptor kinase (MuSK) activity as identified by a screening method described herein in the preparation of a medicament for preserving neuromuscular synapses in a subject is envisioned, wherein the medicament is administered in a therapeutically effective amount that increases MuSK activity in the subject and thereby preserves neuromuscular synapses in the subject.

Yet another embodiment encompasses an agent that increases muscle specific receptor kinase (MuSK) activity as identified by a screening method described herein for use in delaying motor dysfunction in a subject, wherein the agent is administered to the subject in a therapeutically effective amount sufficient to increase MuSK activity in the subject and thereby improve motor function in the subject.

A further embodiment encompasses an agent that increases muscle specific receptor kinase (MuSK) activity as identified by a screening method described herein for use in preserving neuromuscular synapses in a subject, wherein the agent is administered to the subject in a therapeutically effective amount sufficient to increase MuSK activity in the subject and thereby preserve neuromuscular synapses in the subject.

Other objects and advantages will become apparent to those skilled in the art from a review of the following description which proceeds with reference to the following illustrative drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows that MuSK over-expression delays the onset and reduces the extent of denervation in SOD1G93A mice. A) Denervation becomes evident at P120 in SOD1G93A mice and at P130 in SOD1G93A mice over-expressing MuSK. The extent of denervation remains lower in mice over-expressing MuSK through P150. B) At P140, only 8% of synapses are denervated in SOD1G93A mice over-expressing MuSK, whereas 52% of synapses are denervated in SOD1G93A mice. C) Images of synapses from P140 mice stained with α -BGT to label AChRs and antibodies to Neurofilament and Synapsin to label motor axons and nerve terminals. Staining for Synapsin and AChRs overlap at innervated synapses, whereas Synapsin staining is absent from denervated synapses, which retain a high density of AChRs.

FIG. 2 shows that MuSK over-expression preserves full innervation in SOD1G93A mice. A) In SOD1G93A mice, the number of normally innervated synapses falls to 20% by P140. In SOD1G93A mice over-expressing MuSK, nearly 80% of synapses remain normally innervated at P140. B) Normally innervated, partially innervated and denervated synapses are distinguished by differences in overlap of staining for Synapsin and AChRs.

FIG. 3 shows that grip strength and Rota Rod performance are enhanced in SOD1G93A mice over-expressing MuSK. A) The ability of SOD1G93A mice to cling to the wire grid declines rapidly after P110. MuSK over-expression increases the time that mice cling to the wire grid. B) SOD1G93A mice fall from the Rota Rod beginning at P100 whereas mice over-expressing MuSK begin to fall at P120. SOD1G93A mice over-expressing MuSK remain on the rotating bar longer than SOD1G93A mice at all subsequent times. The analysis included 25-30 mice at P80-P120, 35 mice at P130, 40 mice at P140, 32 mice at P150 and 32 at P160. Significantly different values ($p < 0.05$) for SOD1G93A and SOD1G93A; HSA::MuSK-L mice are indicated (*).

FIG. 4 shows that MuSK over-expression does not increase longevity of SOD1G93A mice. A) The mean survival of SOD1G93A; HSA::MuSK-L mice was 164.82 ± 1.83 days,

which is not significantly longer ($p > 0.05$) than SOD1G93A mice (160.1 ± 2.09 days). B) Model for stabilizing motor nerve terminals. In SOD1G93A mice, synapses become denervated as motor neurons lose expression of receptors for muscle retrograde signals, which promote differentiation and muscle-attachment of nerve terminals. In SOD1G93A mice over-expressing MuSK, retrograde signaling is enhanced, preserving nerve terminal differentiation and muscle-attachment at times when receptor expression is reduced but not absent.

FIG. 5 shows that MuSK over-expression delays the onset and reduces the extent of denervation in SOD1G93A mice. A) Immunohistochemistry of the diaphragm muscle stained with antibodies against Synapsin to label nerve terminals and with α -bungarotoxin (α -BGT) to visualize acetylcholine receptors (AChRs) in muscle is shown. The extent of denervation (arrowheads) in SOD1G93A transgenic mice increases progressively from P110 through P150. MuSK over-expression delays the onset and decreases the extent of denervation in SOD1G93A; HSA::MuSK-L transgenic mice. Arrowhead mark denervated synapses. B) Histogram depicting % denervation relative to age.

FIG. 6 demonstrates that copy number of the SOD1G93A transgene did not change over multiple generations. The copy number of the SOD1G93A transgene was measured by real time PCR, which revealed that the copy number did not change over multiple generations and during the course of the experiments.

FIG. 7 reveals that MuSK agonist antibodies #13 and #22 stimulate MuSK phosphorylation. Antibody #22, which reacts with MuSK, and a negative control antibody (g-gD) fail to stimulate MuSK. C2 myotubes were treated with Agrin (1 nM) or antibodies to MuSK (50 nM) for the indicated times. MuSK was immunoprecipitated from lysates, and Western blots were probed with rabbit antibodies to MuSK or phosphotyrosine, as described previously (Friese et al., 2007; Halllock et al., 2010; Herbst and Burden, 2000, the entire content of each of which is incorporated herein by reference).

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al., "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-III [Ausubel, R. M., ed. (1994)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, ed. (1994)]; "Current Protocols in Immunology" Volumes I-III [Coligan, J. E., ed. (1994)]; "Oligonucleotide Synthesis" (M. J. Gait ed. 1984); "Nucleic Acid Hybridization" [B. D. Hames & S. J. Higgins eds. (1985)]; "Transcription And Translation" [B. D. Hames & S. J. Higgins, eds. (1984)]; "Animal Cell Culture" [R. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

A. TERMINOLOGY

The term "specific binding member" describes a member of a pair of molecules, which have binding specificity for one another. The members of a specific binding pair may be naturally derived or wholly or partially synthetically produced. One member of the pair of molecules has an area on its

surface, or a cavity, which specifically binds to and is therefore complementary to a particular spatial and polar organization of the other member of the pair of molecules. Thus the members of the pair have the property of binding specifically to each other. Examples of types of specific binding pairs are antigen-antibody, biotin-avidin, hormone-hormone receptor, receptor-ligand, enzyme-substrate. This application is concerned with antigen-antibody type reactions.

The term "antibody" describes an immunoglobulin whether natural or partly or wholly synthetically produced. The term also covers any polypeptide or protein having a binding domain, which is, or is homologous to, an antibody binding domain. CDR grafted antibodies are also contemplated by this term. An "antibody" is any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term encompasses polyclonal, monoclonal, and chimeric antibodies, the last mentioned described in further detail in U.S. Pat. Nos. 4,816,397 and 4,816,567. The term "antibody(ies)" includes a wild type immunoglobulin (Ig) molecule, generally comprising four full length polypeptide chains, two heavy (H) chains and two light (L) chains, or an equivalent Ig homologue thereof (e.g., a camelid nanobody, which comprises only a heavy chain); including full length functional mutants, variants, or derivatives thereof, which retain the essential epitope binding features of an Ig molecule, and including dual specific, bispecific, multispecific, and dual variable domain antibodies; Immunoglobulin molecules can be of any class (e.g., IgG, IgE, IgM, IgD, IgA, and IgY), or subclass (e.g., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2). Also included within the meaning of the term "antibody" is any "antibody fragment".

An "antibody fragment" means a molecule comprising at least one polypeptide chain that is not full length, including (i) a Fab fragment, which is a monovalent fragment consisting of the variable light (VL), variable heavy (VH), constant light (CL) and constant heavy 1 (CH1) domains; (ii) a F(ab')₂ fragment, which is a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a heavy chain portion of an Fab (Fd) fragment, which consists of the VH and CH1 domains; (iv) a variable fragment (Fv) fragment, which consists of the VL and VH domains of a single arm of an antibody, (v) a domain antibody (dAb) fragment, which comprises a single variable domain (Ward, E. S. et al., Nature 341, 544-546 (1989)); (vi) a camelid antibody; (vii) an isolated complementarity determining region (CDR); (viii) a Single Chain Fv (ScFv) Fragment wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird et al, Science, 242, 423-426, 1988; Huston et al, PNAS USA, 85, 5879-5883, 1988); (ix) a diabody, which is a bivalent, bispecific antibody in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with the complementarity domains of another chain and creating two antigen binding sites (WO94/13804; P. Holliger et al Proc. Natl. Acad. Sci. USA 90 6444-6448, (1993)); and (x) a linear antibody, which comprises a pair of tandem Fv segments (VH-CH1-VH-CH1) which, together with complementarity light chain polypeptides, form a pair of antigen binding regions; (xi) multivalent antibody fragments (scFv dimers, trimers and/or tetramers (Power and Hudson, J. Immunol. Methods 242: 193-204 9 (2000)); and (xii) other non-full length portions of heavy and/or light chains, or mutants, variants, or derivatives thereof, alone or in any combination.

As antibodies can be modified in a number of ways, the term "antibody" should be construed as covering any specific binding member or substance having a binding domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or wholly or partially synthetic. Chimeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023 and U.S. Pat. Nos. 4,816,397 and 4,816,567.

An "antibody combining site" is that structural portion of an antibody molecule comprised of light chain or heavy and light chain variable and hypervariable regions that specifically binds antigen.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. Variability is not, however, evenly distributed throughout antibody variable domains and is concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions which are found in both the light chain and heavy chain variable domains. The more highly conserved portions of variable domains are referred to as the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a beta-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site of an antibody. The constant domains are not involved directly in antigen binding, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule.

Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contain the paratope, including those portions known in the art as Fab, Fab', F(ab')₂ and F(v), which portions are preferred for use in the therapeutic methods described herein.

Antibodies may also be bispecific, wherein one binding domain of the antibody is a specific binding member of the invention, and the other binding domain has a different specificity, e.g. to recruit an effector function or the like. Bispecific antibodies of the present invention include wherein one binding domain of the antibody is a specific binding member of the present invention, including a fragment thereof, and the other binding domain is a distinct antibody or fragment thereof, including that of a distinct anti-growth hormone receptor specific antibody. The other binding domain may be an antibody that recognizes or targets a particular cell type, as in a muscle, neural or glial cell-specific antibody. In the bispecific antibodies of the present invention the one binding domain of the antibody of the invention may be combined with other binding domains or molecules which recognize particular cell receptors and/or modulate cells in a particular fashion, as for instance an immune modulator (e.g., interleukin(s)), a growth modulator or cytokine, or mitotic agent or factor.

The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may also contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen; e.g., a bispecific (chimeric) monoclonal antibody.

The term "antigen binding domain" describes the part of an antibody which comprises the area which specifically binds to and is complementary to part or all of an antigen. Where an antigen is large, an antibody may bind to a particular part of the antigen only, which part is termed an epitope. An antigen binding domain may be provided by one or more antibody variable domains. Preferably, an antigen binding domain comprises an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH).

The term "specific" may be used to refer to the situation in which one member of a specific binding pair will not show any significant binding to molecules other than its specific binding partner(s). The term is also applicable where e.g. an antigen binding domain is specific for a particular epitope which is carried by a number of antigens, in which case the specific binding member carrying the antigen binding domain will be able to bind to the various antigens carrying the epitope.

The term "activating antibody" refers an antibody that upon binding specifically to an antigen for which it has specificity activates the antigen. With respect to an activating antibody specific for MuSK, upon binding MuSK, the activating antibody would activate MuSK, thus leading to MuSK phosphorylation and subsequent clustering of AChR. Exemplary such antibodies include agonist antibodies #13 and #22 described herein. See also Xie et al. (1997), the entire content of which is incorporated herein in its entirety.

The term "adjuvant" refers to a compound or mixture that enhances the immune response, particularly to an antigen. An adjuvant can serve as a tissue depot that slowly releases the antigen and also as a lymphoid system activator that non-specifically enhances the immune response (Hood et al., *Immunology, Second Ed.*, 1984, Benjamin/Cummings: Menlo Park, Calif., p. 384). Often, a primary challenge with an antigen alone, in the absence of an adjuvant, will fail to elicit a humoral or cellular immune response. Previously known and utilized adjuvants include, but are not limited to, complete Freund's adjuvant, incomplete Freund's adjuvant, saponin, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvant such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*. Mineral salt adjuvants include but are not limited to: aluminum hydroxide, aluminum phosphate, calcium phosphate, zinc hydroxide and calcium hydroxide. Preferably, the adjuvant composition further comprises a lipid of fat emulsion comprising about 10% (by weight) vegetable oil and about 1-2% (by weight) phospholipids. Preferably, the adjuvant composition further optionally comprises an emulsion form having oily particles dispersed in a continuous aqueous phase, having an emulsion forming polyol in an amount of from about 0.2% (by weight) to about 49% (by weight), optionally a metabolizable oil in an emulsion-forming amount of up to 15% (by weight), and optionally a glycol ether-based surfactant in an emulsion-stabilizing amount of up to about 5% (by weight).

As used herein, the term "immunomodulator" refers to an agent which is able to modulate an immune response. An example of such modulation is an enhancement of cell activation or of antibody production.

The term "effective amount" of an immunomodulator refers to an amount of an immunomodulator sufficient to enhance an immune response (e.g., a vaccine-induced immune response), be it cell-mediated, humoral or antibody-mediated. An effective amount of an immunomodulator, if injected, can be in the range of about 0.1-1,000 µg, preferably 1-900 µg, more preferably 5-500 µg, for a human subject, or in the range of about 0.01-10.0 µg/Kg body weight of the subject animal. This amount may vary to some degree depending on the mode of administration, but will be in the same general range. If more than one immunomodulator is used, each one may be present in these amounts or the total amount may fall within this range. An effective amount of an antigen may be an amount capable of eliciting a demonstrable immune response in the absence of an immunomodulator. For many antigens, this is in the range of about 5-100 µg for a human subject. The appropriate amount of antigen to be used is dependent on the specific antigen and is well known in the art.

The exact effective amount necessary will vary from subject to subject, depending on the species, age and general condition of the subject, the severity of the condition being treated, the mode of administration, etc. Thus, it is not possible to specify an exact effective amount. However, the appropriate effective amount may be determined by one of ordinary skill in the art using only routine experimentation or prior knowledge in the vaccine art.

An "immunological response" to a composition or vaccine comprised of an antigen is the development in the host of a cellular- and/or antibody-mediated immune response to the composition or vaccine of interest. Usually, such a response consists of the subject producing antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells directed specifically to an antigen or antigens included in the composition or vaccine of interest.

The term "comprise" is generally used in the sense of include, that is to say permitting the presence of one or more features or components.

The term "consisting essentially of" refers to a product, particularly a peptide sequence, of a defined number of residues which is not covalently attached to a larger product. In the case of the peptide of the invention referred to above, those of skill in the art will appreciate that minor modifications to the N- or C-terminal of the peptide may however be contemplated, such as the chemical modification of the terminal to add a protecting group or the like, e.g. the amidation of the C-terminus.

The term "isolated" refers to the state in which specific binding members of the invention or nucleic acid encoding such binding members will be, in accordance with the present invention. Members and nucleic acid will be free or substantially free of material with which they are naturally associated such as other polypeptides or nucleic acids with which they are found in their natural environment, or the environment in which they are prepared (e.g. cell culture) when such preparation is by recombinant DNA technology practiced in vitro or in vivo. Members and nucleic acid may be formulated with diluents or adjuvants and still for practical purposes be isolated—for example the members will normally be mixed with gelatin or other carriers if used to coat microtitre plates for use in immunoassays, or will be mixed with pharmaceutically acceptable carriers or diluents when used in diagnosis or therapy.

As used herein, "pg" means picogram, "ng" means nanogram, "ug" or "μg" mean microgram, "mg" means milligram, "ul" or "μl" mean microliter, "ml" means milliliter, "l" means liter.

The amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property of immunoglobulin-binding is retained by the polypeptide. NH₂ refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J. Biol. Chem.*, 243:3552-59 (1969), abbreviations for amino acid residues are shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE		
SYMBOL		
1-Letter	3-Letter	AMINO ACID
Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine
M	Met	methionine
A	Ala	alanine
S	Ser	serine
I	Ile	isoleucine
L	Leu	leucine
T	Thr	threonine
V	Val	valine
P	Pro	proline
K	Lys	lysine
H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine
C	Cys	cysteine

It should be noted that all amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues. The above Table is presented to correlate the three-letter and one-letter notations which may appear alternately herein.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication in vivo; i.e., capable of replication under its own control.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction

along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

An "origin of replication" refers to those DNA sequences that participate in DNA synthesis.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease Si), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

A "signal sequence" can be included before the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

The term "oligonucleotide," as used herein in referring to the probe of the present invention, is defined as a molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired

extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith and thereby form the template for the synthesis of the extension product.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transformed" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth in vitro for many generations.

Two DNA sequences are "substantially homologous" when at least about 75% (preferably at least about 80%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis et al., *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

It should be appreciated that also within the scope of the present invention are DNA sequences encoding protein or peptide sequences as provided herein, or comprising sequences which are degenerate thereto. DNA sequences having the nucleic acid sequence encoding the peptides of the invention are contemplated, including degenerate sequences thereof encoding the same, or a conserved or substantially similar, amino acid sequence. By "degenerate to" is meant that a different three-letter codon is used to specify a particular amino acid. It is well known in the art that the following codons can be used interchangeably to code for each specific amino acid:

Phenylalanine (Phe or F)	UUU or UUC
Leucine (Leu or L)	UUA or UUG or CUU or CUC or CUA or CUG
Isoleucine (Ile or I)	AUU or AUC or AUA
5 Methionine (Met or M)	AUG
Valine (Val or V)	GUU or GUC or GUA or GUG
Serine (Ser or S)	UCU or UCC or UCA or UCG or AGU or AGC
Proline (Pro or P)	CCU or CCC or CCA or CCG
Threonine (Thr or T)	ACU or ACC or ACA or ACG
10 Alanine (Ala or A)	GCU or GCG or GCA or GCG
Tyrosine (Tyr or Y)	UAU or UAC
Histidine (His or H)	CAU or CAC
Glutamine (Gln or Q)	CAA or CAG
Asparagine (Asn or N)	AAU or AAC
Lysine (Lys or K)	AAA or AAG
15 Aspartic Acid (Asp or D)	GAU or GAC
Glutamic Acid (Glu or E)	GAA or GAG
Cysteine (Cys or C)	UGU or UGC
Arginine (Arg or R)	CGU or CGC or CGA or CCG or AGA or AGG
Glycine (Gly or G)	GGU or GGC or GGA or GGG
20 Tryptophan (Trp or W)	UGG
Termination codon	UAA (ochre) or UAG (amber) or UGA (opal)

It should be understood that the codons specified above are for RNA sequences. The corresponding codons for DNA have a T substituted for U.

Mutations can be made in the sequences encoding the protein or peptide sequences of the invention, such that a particular codon is changed to a codon which codes for a different amino acid. Such a mutation is generally made by making the fewest nucleotide changes possible. A substitution mutation of this sort can be made to change an amino acid in the resulting protein in a non-conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping). Such a conservative change generally leads to less change in the structure and function of the resulting protein. A non-conservative change is more likely to alter the structure, activity or function of the resulting protein. The present invention should be considered to include sequences containing conservative changes which do not significantly alter the activity or binding characteristics of the resulting protein.

The following is one example of various groupings of amino acids:

Amino Acids with Nonpolar R Groups

Alanine, Valine, Leucine, Isoleucine, Proline, Phenylalanine, Tryptophan, Methionine

Amino Acids with Uncharged Polar R Groups

Glycine, Serine, Threonine, Cysteine, Tyrosine, Asparagine, Glutamine

Amino Acids with Charged Polar R Groups (Negatively Charged at pH 6.0)

Aspartic acid, Glutamic acid

Basic Amino Acids (Positively Charged at pH 6.0)

Lysine, Arginine, Histidine (at pH 6.0)

Another grouping may be those amino acids with phenyl groups:

Phenylalanine, Tryptophan, Tyrosine

Another grouping may be according to molecular weight (i.e., size of R groups):

Glycine	75	Alanine	89
Serine	105	Proline	115
Valine	117	Threonine	119
Cysteine	121	Leucine	131
Isoleucine	131	Asparagine	132
Aspartic acid	133	Glutamine	146
Lysine	146	Glutamic acid	147
Methionine	149	Histidine (at pH 6.0)	155
Phenylalanine	165	Arginine	174
Tyrosine	181	Tryptophan	204

Particularly preferred substitutions are:

Lys for Arg and vice versa such that a positive charge may be maintained;

Glu for Asp and vice versa such that a negative charge may be maintained;

Ser for Thr such that a free —OH can be maintained; and

Gln for Asn such that a free NH₂ can be maintained.

Exemplary and preferred conservative amino acid substitutions include any of: glutamine (Q) for glutamic acid (E) and vice versa; leucine (L) for valine (V) and vice versa; serine (S) for threonine (T) and vice versa; isoleucine (I) for valine (V) and vice versa; lysine (K) for glutamine (Q) and vice versa; isoleucine (I) for methionine (M) and vice versa; serine (S) for asparagine (N) and vice versa; leucine (L) for methionine (M) and vice versa; lysine (L) for glutamic acid (E) and vice versa; alanine (A) for serine (S) and vice versa; tyrosine (Y) for phenylalanine (F) and vice versa; glutamic acid (E) for aspartic acid (D) and vice versa; leucine (L) for isoleucine (I) and vice versa; lysine (K) for arginine (R) and vice versa.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly “catalytic” site (i.e., His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces beta-turns in the protein's structure.

Two amino acid sequences are “substantially homologous” when at least about 70% of the amino acid residues (preferably at least about 80%, and most preferably at least about 90 or 95%) are identical, or represent conservative substitutions.

A “heterologous” region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

A DNA sequence is “operatively linked” to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that DNA sequence. The term “operatively linked” includes having an appropriate start signal (e.g., ATG) in front of the DNA sequence to be expressed and maintaining the correct reading frame to permit expression of the DNA sequence under the control of the expression control sequence and production of the desired product encoded by the DNA sequence. If a gene that one desires to insert into a recombinant DNA molecule

does not contain an appropriate start signal, such a start signal can be inserted in front of the gene.

The term “standard hybridization conditions” refers to salt and temperature conditions substantially equivalent to 5×SSC and 65° C. for both hybridization and wash. However, one skilled in the art will appreciate that such “standard hybridization conditions” are dependent on particular conditions including the concentration of sodium and magnesium in the buffer, nucleotide sequence length and concentration, percent mismatch, percent formamide, and the like. Also important in the determination of “standard hybridization conditions” is whether the two sequences hybridizing are RNA-RNA, DNA-DNA or RNA-DNA. Such standard hybridization conditions are easily determined by one skilled in the art according to well known formulae, wherein hybridization is typically 10-20° C. below the predicted or determined T_m with washes of higher stringency, if desired.

The term ‘agent’ means any molecule, including polypeptides, antibodies, polynucleotides, chemical compounds and small molecules. In particular the term agent includes compounds such as test compounds or drug candidate compounds.

The term ‘agonist’ refers to a ligand that stimulates the receptor the ligand binds to in the broadest sense or stimulates a response that would be elicited on binding of a natural binder to a binding site.

The term ‘assay’ means any process used to measure a specific property of a compound or agent. A ‘screening assay’ means a process used to characterize or select compounds based upon their activity from a collection of compounds.

The term ‘preventing’ or ‘prevention’ refers to a reduction in risk of acquiring or developing a disease or disorder (i.e., causing at least one of the clinical symptoms of the disease not to develop) in a subject that may be exposed to a disease-causing agent, or predisposed to the disease in advance of disease onset.

The term ‘prophylaxis’ is related to and encompassed in the term ‘prevention’, and refers to a measure or procedure the purpose of which is to prevent, rather than to treat or cure a disease. Non-limiting examples of prophylactic measures may include the administration of vaccines; the administration of low molecular weight heparin to hospital patients at risk for thrombosis due, for example, to immobilization; and the administration of an anti-malarial agent such as chloroquine, in advance of a visit to a geographical region where malaria is endemic or the risk of contracting malaria is high.

‘Subject’ includes humans. The terms “human,” “patient” and “subject” may be used interchangeably herein.

‘Therapeutically effective amount’ means that amount of a drug, compound, antimicrobial, antibody, or pharmaceutical agent that will elicit the biological or medical response of a subject that is being sought by a medical doctor or other clinician. As an example, with regard to immune response, the term “effective amount” is intended to include an effective amount of a compound or agent that will bring about a biologically meaningful decrease in the amount of or extent of immune response, activation indicator and/or a biologically meaningful increase in the amount or extent of dendritic cell, T cell and/or B cell effects. The phrase “therapeutically effective amount” is used herein to mean an amount sufficient to prevent, and preferably reduce by at least about 30 percent, more preferably by at least 50 percent, most preferably by at least 90 percent, a clinically significant change in the immune response or immune cell indicator or response, or in a patient's response to an antigen, vaccine, or other immune agent, or in a patient's clearance of an infectious agent, or

other feature of pathology such as for example, elevated activated T or B cells, activated DC cell count, fever or white cell count.

The term 'treating' or 'treatment' of any disease or infection refers, in one embodiment, to ameliorating the disease or infection (i.e., arresting the disease or growth of the infectious agent or bacteria or reducing the manifestation, extent or severity of at least one of the clinical symptoms thereof). In another embodiment 'treating' or 'treatment' refers to ameliorating at least one physical parameter, which may not be discernible by the subject. In yet another embodiment, 'treating' or 'treatment' refers to modulating the disease or infection, either physically (e.g., stabilization of a discernible symptom), physiologically, (e.g., stabilization of a physical parameter), or both. In a further embodiment, 'treating' or 'treatment' relates to slowing the progression of a disease or reducing an infection.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human.

B. DETAILED DISCLOSURE

Previous investigations have shown that neuromuscular synapses form in mice lacking Agrin, but these synapses are unstable, leading to axon withdrawal and neonatal lethality. The present inventor and his colleagues found that a three-fold increase in MuSK expression, conferred by a MuSK-L transgene, rescued these neuromuscular synapses and prevented the neonatal lethality of agrin mutant mice (Kim and Burden, 2008). These studies demonstrated that a modest increase in MuSK activity is sufficient to prevent the disassembly of neuromuscular synapses that occurs in the absence of Agrin.

The experiments described herein were performed to determine whether MuSK over-expression might prevent motor axon withdrawal in other mechanistically distinct instances of synaptic dysfunction. To address this issue, the present inventor crossed the MuSK transgene into a mouse model of ALS (SOD1G93A mice), wherein mice die prematurely due to motor axon withdrawal from muscle and ultimately motor neuron death. In experiments described herein, the present inventor surprisingly found that MuSK over-expression delays muscle denervation by forty days, preserving motor behavior that would otherwise be severely compromised in SOD1G93A mice. These results suggest that increasing MuSK activity might delay symptoms and therefore be beneficial for patients with ALS.

The finding that MuSK over-expression could partially rescue SOD1G93A mice was surprising for a variety of reasons. To begin, ALS is a motor neuron disease, whereas MuSK is expressed in muscle, not motor neurons. Thus, it is surprising that over-expression of any protein in a cell type (i.e., muscle cell) other than the primarily impaired cell type could compensate, even partially, for the genetic defects of the primary cell type involved in ALS. Moreover, motor neurons in ALS are compromised in numerous ways. The deficiencies in, for example, mitochondrial function and axonal transport in ALS motor neurons compromise motor neuron function and activity so severely that it could not be envisioned with any degree of likelihood that synapse preservation could be preserved by increased expression of any single protein. This is especially apparent with respect to increasing expression of a protein in cells other than motor neurons wherein the activity of the protein can have no direct effect on motor neuron activity/

function. Given the above, it is surprising that over-expression of MuSK in muscle cells can preserve neuromuscular synapses in the context of a genetic background characterized by motor neurons having severely impaired activity/function.

As described herein, the present inventor crossed MuSK-L mice with SOD1G93A mice, which generated four types of progeny: (1) wild-type mice; (2) MuSK-L; (3) SOD1G93A; (4) MuSK-L; SOD1G93A mice. The longevity of these mice was assessed and MuSK over-expression, such as that observed in MuSK-L mice, did not significantly prolong the longevity of SOD1G93A mice. However, MuSK over-expression substantially delayed the onset and decreased the extent of muscle denervation in MuSK-L; SOD1G93A mice relative to SOD1G93A mice, as reflected by the observation that substantially more neuromuscular synapses appeared intact over a forty day period (P120-P160) when synapses disassemble in SOD1G93A mice. Consistent with these histological findings, MuSK over-expression substantially improved motor function of SOD1G93A mice in that MuSK-L; SOD1G93A mice exhibited greater ambulatory behavior and muscle strength, as assessed on a rotarod and by grip strength, over the same 40 day period, when SOD1G93A mice showed severe signs of motor dysfunction and muscle weakness.

The present findings indicate that increasing MuSK activity in muscle is sufficient to delay the onset and decrease the extent of synaptic loss and improve motor dysfunction in a mouse model of ALS. As such, increasing MuSK activity in humans with ALS has the potential to alleviate and delay motor dysfunction, allowing ALS patients to function without assistance for a more prolonged period of time.

To pursue these findings further, studies are ongoing to evaluate the ability of other means to activate MuSK in vivo to alleviate motor dysfunction in ALS patients. Several possibilities for MuSK activators having potential utility in in vivo applications include, without limitation: (1) Agrin and Lrp4, which are known MuSK activators and can be tested in the mouse model described herein; (2) Antibodies that activate MuSK in cultured muscle [see Xie et al. Nat. Biotech. 1997 15:768] and can be tested in vivo in, for example, the mouse model described herein; and (3) using small molecule activators of MuSK identified in the screening assays described herein. Fragments thereof, such as ScFv can also be tested using similar and/or identical assays.

Moreover, these findings raise the possibility that increasing MuSK activity may alleviate motor dysfunction in other disorders associated with nerve terminal loss or fragmentation, irrespective of the underpinning mechanistic causes that led to the nerve terminal loss or fragmentation. Such disorders include, for example, sarcopenia. Increasing MuSK activity may also improve motor function in auto-immune, anti-MuSK myasthenia gravis.

Amyotrophic Lateral Sclerosis

ALS is a devastating neurodegenerative disease, culminating in the death of motor neurons, complete muscle paralysis and lethal respiratory failure, without cognitive impairment, within five years of diagnosis (Fischer et al., 2004; Pasinelli and Brown, 2006; Schaefer et al., 2005). The incidence of disease is ~1/7,500, similar to muscular dystrophy or myasthenia gravis. The mechanisms responsible for neuronal cell death are poorly understood, and studies designed to inhibit motor neuron cell death, either by blocking cell death pathways or providing broadly acting growth factors, have had little impact on disease progression (Kostic et al., 1997; Pun et al., 2006; Sagot et al., 1995). As such, there are no therapies to cure let alone slow disease progression.

Dominant mutations in SOD1, TDP-43, FUS, and the recently discovered C9orf72 gene are responsible for familial forms of ALS and together represent ~17% of all cases of ALS (Chen-Plotkin et al., 2010; Kwiatkowski et al., 2009; Majounie et al., 2012; Pasinelli and Brown, 2006; Renton et al., 2011; Vance et al., 2009). The pathological hallmarks of the disease are well replicated in mice that overexpress mutant forms of SOD1 in motor neurons, providing an excellent model system for studying the pathology of ALS and identifying approaches to treat this devastating disease (Pasinelli and Brown, 2006). Although cell types other than motor neurons, including grey matter oligodendrocytes and microglia contribute to disease onset and progression (Ilieva et al., 2009; Kang et al., 2010), expression of mutant forms of SOD1 in skeletal muscle does not cause disease (Miller et al., 2006). As such, dominant mutations in SOD1 act largely in an autonomous manner within motor neurons, consistent with the idea that ALS is primarily a disease of upper and lower motor neurons.

Sarcopenia

Sarcopenia, the atrophy and deterioration of skeletal muscle that leads to weakness and fatigue, is a major and debilitating consequence of aging. Moreover, sarcopenia is a major driving force of aging, as sarcopenia reduces physical activity and thereby compromises health by contributing to a wide range of complications in multiple organ systems, including vascular, cardiac, pulmonary and skeletal systems. The factors that trigger sarcopenia are poorly understood, but most attention has been focused on the idea that an increase in proteasome activity in muscle causes an increase in protein breakdown and causes muscle atrophy. As such, sarcopenia is largely considered a muscle disease, not a synaptic disease. Myasthenia Gravis

Myasthenia gravis is an auto-immune disease caused by auto-antibodies to synaptic proteins, including acetylcholine receptors (AChRs), MuSK & Lrp4, leading to muscle weakness. The prevalence in the US is 1/~7,500. Auto-antibodies to AChRs stimulate accelerated degradation of AChRs and cause complement-mediated structural disorganization of the synapse. In contrast, auto-antibodies to MuSK are IgG4, which are functionally monovalent and fail to engage complement, suggesting that they interfere with MuSK function, rather than recruiting complement. Our recent, unpublished studies, in collaboration with the Verschuuren lab in Leiden, Netherlands, show that disease-causing auto-antibodies to MuSK interfere with the ability of MuSK to bind Lrp4, and therefore provide a ready explanation for the inhibitory effects of these antibodies.

Therapeutic Strategies

Several types of therapeutic strategies are envisioned: (1) Since Agrin is an activator of MuSK, supplying Agrin may prove therapeutic in patients with ALS. As indicated above, the SOD1G93A mouse model described herein is a suitable screening system in which to investigate therapeutic efficacy of agrin and agrin biological mimetics. (2) Since Lrp4 binds and activates MuSK, fragments of Lrp4 that bind MuSK or mimics of Lrp4 may prove therapeutic in patients with ALS. As indicated above, the SOD1G93A mouse model described herein can be used as an initial screening system to test efficacy. (3) Antibodies to Lrp4 or MuSK that stimulate MuSK may prove therapeutic in patients with ALS. This idea could be first tested using the SOD1G93A mouse model described here. (4) Small molecules that activate MuSK may prove therapeutic in patients with ALS. Such activators might be identified by screening for small molecules that stimulate MuSK phosphorylation in cultured cells.

Agrin is a large heparin sulphate proteoglycan with multiple domains, which is located in the extracellular matrix. It is involved in neuromuscular junction formation, wherein it directs clustering of postsynaptic molecules, including acetylcholine receptors (AChRs). This activity is conferred by the C-terminal portion of the protein, which consists of three laminin-like globular domains (G-domains: G1, G2 and G3) and four EGF-like repeats. In a study to investigate the contributions of individual domains and alternate splicing to agrin activity, single G-domains and covalently linked pairs of G-domains were expressed as soluble proteins and their AChR clustering activity assessed using cultured C2 myotubes. See Cornish et al. (J Cell Sci 1999 112:1213-1223), the entire content of which is incorporated herein by reference in its entirety.

This study revealed that only G3(8) exhibits detectable activity by itself, but all G-domains studied (G1, G2(0), G2(4), G3(0) and G3(8)) enhance G3(8) activity when physically linked to G3(8). This effect is most pronounced when G2(4) is linked to G3(8) and is independent of the order of the G-domains. The study also revealed that deletion of EGF-like repeats enhances activity and increasing the physical separation between linked G1 and G3(8) domains produces a significant increase in activity, whereas similar alterations to linked G2 and G3(8) domains are without effect. Clusters induced by two concatenated G3(8) domains were also shown to be significantly smaller than all other agrin forms studied. The study concluded that agrin G-domains are the functional units involved in AChR clustering and interact independently of their specific organization in this activity. See Cornish et al. (J Cell Sci 1999 112:1213-1223).

As described in Patel et al. (Protein Sci 2011 20:931-9400, the entire content of which is incorporated herein by reference in its entirety), the C-terminal G3 domain of agrin is critical for agrin function since it harbors an α -dystroglycan binding site and carries out acetylcholine receptor clustering activities. To further investigate its functionality, Patel et al. fused the G3 domain of agrin to an IgG Fc domain to produce a G3-Fc fusion protein. As further described therein, the G3-Fc protein forms a T-shaped molecule with the G3 domains extruding perpendicularly from the Fc scaffold. In light of the functional significance of the G3 domain, the G3-Fc fusion protein of Patel et al. and structurally similar fusion protein are exemplary agents to be tested in the screening assays described herein.

Accordingly, human agrin and functional fragments thereof are exemplary agents to be tested in the screening assays described herein. Human agrin and functional fragments, such as, for example, G3(8) alone or linked to G1, G2(0), G2(4), G3(0) and/or G3(8), are also envisioned as exemplary agents for use as MuSK activators in applications pertaining to methods for treating subjects in need thereof and for use in delaying motor dysfunction in a subject in need thereof, or for use in preserving neuromuscular synapses in a subject in need thereof. Exemplary nucleic and amino acid sequences are known in the art (see, e.g., GenBank Accession Number NM_198576.3 for human agrin mRNA) and presented herein as SEQ ID NOs: 1 and 2, respectively.

Further to the above, human Lrp4 and functional fragments thereof are also exemplary agents to be tested in the screening assays described herein. Human Lrp4 and functional fragments are also envisioned as exemplary agents for use as MuSK activators in applications pertaining to methods for treating subjects in need thereof and for use in delaying motor dysfunction in a subject in need thereof, or for use in preserving neuromuscular synapses in a subject in need thereof.

Exemplary nucleic and amino acid sequences are known in the art (see, e.g., GenBank Accession Number NM_002334.33 for human Lrp4 mRNA) and presented herein as SEQ ID NOs: 3 and 4, respectively.

Additional exemplary agents envisioned for testing in the screening assays described herein include activating antibodies immunospecific for MuSK. Xie et al. (Nat. Biotech. 1997 15:768), for example, describe monovalent scFv activating antibodies, as well as IgG counterparts of these antibodies to MuSK. In light of the above, it is clearly possible to screen and identify antibodies that are capable of acting as MuSK agonists. The content of Xie et al. (Nat. Biotech. 1997 15:768) is incorporated herein by reference in its entirety.

In accordance with standard practice in antibody technology, it is understood that full length antibodies or antigen binding fragments or variants thereof can be envisioned that comprise variable heavy (VH) and variable light (VL) chains of exemplary scFv fragments. It is, moreover, understood that some variation in framework sequences is tolerated and does not necessarily alter the binding properties or functional activity of antibodies or functional fragments or variants thereof as long as the CDR sequences, which are critical for binding specificity, are preserved. Functional properties of different regions of antibodies and functional fragments and variants thereof are known to those skilled in the art and described in, for example, Kabat et al., *Sequences of Proteins of Immunological Interest*, National Institutes of Health, Bethesda, Md. (1987).

Additional information pertaining to the exemplary scFvs described, including nucleic acid sequences encoding same and methods of making same, can be found, for example, in the Sequence Listing and Examples of U.S. Pat. No. 6,737,249 and United States Patent Application Publication No. 2006/0177441, the entire content of each of which is incorporated herein by reference.

In one embodiment, screening assays described herein would require growing mouse muscle cell lines, such as, for example, C2 cells, and allowing the cells to differentiate to form myotubes. This is very straightforward. Cells would be plated in 96 well plates and treated with small molecule libraries. Since MuSK phosphorylation leads to clustering of acetylcholine receptors (AChR), which can be readily visualized by fluorescent microscopy, measuring AChR clustering would be the simplest means to identify small molecules that activate MuSK. Moreover, because AChR clustering is exceedingly rare in the absence of MuSK activation, it should be quite simple to distinguish a bona fide signal from noise. This primary assay would be followed-up with a secondary assay in which MuSK phosphorylation is directly measured by immunoprecipitating MuSK from cell lysates and probing Western blots with antibodies to phosphotyrosine.

Nucleic and amino acid sequences corresponding to MuSK are presented in U.S. Pat. Nos. 5,814,478; 6,413,740; and 6,852,838, the entire contents of each of which is incorporated herein in its entirety.

In one particular embodiment, with respect to the compounds or agents described herein, the compound/agent is administered alone or in conjunction with other compounds/agents described herein or known to be efficacious in the treatment of a disorder associated with synaptic loss and motor dysfunction such as those described herein. In another aspect, pharmaceutical compositions comprising a compound/agent or a plurality of the compounds/agents described herein are administered to a subject in need thereof. Compounds and agents described herein may also be used in the

preparation of a medicament for treating a disorder associated with synaptic loss and motor dysfunction such as those described herein.

In one embodiment, with respect to the method of treatment, use of an agent of compound, or preparation of a medicament, the disease or condition is a disorder associated with synaptic loss and motor dysfunction. Such disorders include amyotrophic lateral sclerosis (ALS), sarcopenia, and anti-MuSK myasthenia gravis. In a particular embodiment, the disease or condition is associated with nerve terminal loss or fragmentation, wherein such condition is amyotrophic lateral sclerosis.

Pharmaceutical Compositions

When employed as pharmaceuticals, the compounds of this invention are typically administered in the form of a pharmaceutical composition. Such compositions can be prepared in a manner well known in the pharmaceutical art and comprise at least one active compound.

Generally, the compounds of this invention are administered in a pharmaceutically effective amount. The amount of the compound actually administered will typically be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual compound administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the like.

The pharmaceutical compositions of this invention can be administered by a variety of routes including oral, rectal, transdermal, subcutaneous, intravenous, intramuscular, and intranasal. Depending on the intended route of delivery, the compounds of this invention are preferably formulated as either injectable or oral compositions or as salves, as lotions or as patches all for transdermal administration.

The compositions for oral administration can take the form of bulk liquid solutions or suspensions, or bulk powders. More commonly, however, the compositions are presented in unit dosage forms to facilitate accurate dosing. The term "unit dosage forms" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient. Typical unit dosage forms include prefilled, premeasured ampules or syringes of the liquid compositions or pills, tablets, capsules or the like in the case of solid compositions. In such compositions, the furansulfonic acid compound is usually a minor component (from about 0.1 to about 50% by weight or preferably from about 1 to about 40% by weight) with the remainder being various vehicles or carriers and processing aids helpful for forming the desired dosing form.

Liquid forms suitable for oral administration may include a suitable aqueous or nonaqueous vehicle with buffers, suspending and dispensing agents, colorants, flavors and the like. Solid forms may include, for example, any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

Injectable compositions are typically based upon injectable sterile saline or phosphate-buffered saline or other injectable carriers known in the art. As before, the active compound in such compositions is typically a minor component, often

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being from about 0.05 to 10% by weight with the remainder being the injectable carrier and the like.

Transdermal compositions are typically formulated as a topical ointment or cream containing the active ingredient(s), generally in an amount ranging from about 0.01 to about 20% by weight, preferably from about 0.1 to about 20% by weight, preferably from about 0.1 to about 10% by weight, and more preferably from about 0.5 to about 15% by weight. When formulated as a ointment, the active ingredients will typically be combined with either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredients may be formulated in a cream with, for example an oil-in-water cream base. Such transdermal formulations are well-known in the art and generally include additional ingredients to enhance the dermal penetration of stability of the active ingredients or the formulation. All such known transdermal formulations and ingredients are included within the scope of this invention.

The compounds of this invention can also be administered by a transdermal device. Accordingly, transdermal administration can be accomplished using a patch either of the reservoir or porous membrane type, or of a solid matrix variety.

The above-described components for orally administrable, injectable or topically administrable compositions are merely representative. Other materials as well as processing techniques and the like are set forth in Part 8 of *Remington's Pharmaceutical Sciences*, 17th edition, 1985, Mack Publishing Company, Easton, Pa., which is incorporated herein by reference.

The compounds of this invention can also be administered in sustained release forms or from sustained release drug delivery systems. A description of representative sustained release materials can be found in *Remington's Pharmaceutical Sciences*.

The following formulation examples illustrate representative pharmaceutical compositions of this invention. The present invention, however, is not limited to the following pharmaceutical compositions.

Formulation 1

Tablets

A compound of the invention is admixed as a dry powder with a dry gelatin binder in an approximate 1:2 weight ratio. A minor amount of magnesium stearate is added as a lubricant. The mixture is formed into 240-270 mg tablets (80-90 mg of active amide compound per tablet) in a tablet press.

Formulation 2

Capsules

A compound of the invention is admixed as a dry powder with a starch diluent in an approximate 1:1 weight ratio. The mixture is filled into 250 mg capsules (125 mg of active amide compound per capsule).

Formulation 3

Liquid

A compound of the invention (125 mg), sucrose (1.75 g) and xanthan gum (4 mg) are blended, passed through a No. 10 mesh U.S. sieve, and then mixed with a previously made solution of microcrystalline cellulose and sodium carboxymethyl cellulose (11:89, 50 mg) in water. Sodium benzoate (10

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mg), flavor, and color are diluted with water and added with stirring. Sufficient water is then added to produce a total volume of 5 mL.

Formulation 4

Tablets

A compound of the invention is admixed as a dry powder with a dry gelatin binder in an approximate 1:2 weight ratio. A minor amount of magnesium stearate is added as a lubricant. The mixture is formed into 450-900 mg tablets (150-300 mg of active amide compound) in a tablet press.

Formulation 5

Injection

A compound of the invention is dissolved or suspended in a buffered sterile saline injectable aqueous medium to a concentration of approximately 5 mg/ml.

Formulation 6

Topical

Stearyl alcohol (250 g) and a white petrolatum (250 g) are melted at about 75° C. and then a mixture of a compound of the invention (50 g) methylparaben (0.25 g), propylparaben (0.15 g), sodium lauryl sulfate (10 g), and propylene glycol (120 g) dissolved in water (about 370 g) is added and the resulting mixture is stirred until it congeals.

Methods of Treatment

The present compounds/agents are used as therapeutic agents for the treatment of conditions in mammals that are associated with synaptic loss and motor dysfunction. Such disorders include ALS, sarcopenia, and myasthenia gravis. Accordingly, the compounds and pharmaceutical compositions of this invention find use as therapeutics for preventing and/or treating a variety of conditions and disorders in mammals, including humans.

In a method of treatment aspect, this invention provides a method of treating a mammal susceptible to or afflicted with a condition or disorder associated with synaptic loss and motor dysfunction, which method comprises administering an effective amount of one or more of the pharmaceutical compositions just described.

In additional method of treatment aspects, this invention provides methods of treating a mammal susceptible to or afflicted with a condition or disorder causally related or attributable to synaptic loss. Such conditions and disorders include, ALS, sarcopenia, and myasthenia gravis. Such methods comprise administering an effective condition-treating or condition-preventing amount of one or more of the pharmaceutical compositions just described. In a particular embodiment thereof, the condition or disorder is ALS, sarcopenia, or anti-MuSK myasthenia gravis. In a more particular embodiment, the condition or disorder is ALS. In an even more particular embodiment, the condition or disorder is sarcopenia.

As a further aspect of the invention, the present compounds are provided for use as pharmaceuticals, especially in the treatment or prevention of the aforementioned conditions and diseases. Also provided herein is the use of the present compounds in the manufacture of a medicament for the treatment or prevention of one of the aforementioned conditions and diseases. Also encompassed is at least one compound or agent

described herein for use in treating or preventing one of the aforementioned conditions and diseases. Further to the above, a combination of one or more of the compounds/agents described herein for a method of treating a condition or disorder causally related or attributable to synaptic loss in a subject is also encompassed herein.

Injection dose levels range from about 0.1 mg/kg/hour to at least 10 mg/kg/hour, all for from about 1 to about 120 hours and especially 24 to 96 hours. A preloading bolus of from about 0.1 mg/kg to about 10 mg/kg or more may also be administered to achieve adequate steady state levels. The maximum total dose is not expected to exceed about 2 g/day for a 40 to 80 kg human patient.

For the prevention and/or treatment of long-term conditions, such as, e.g., ALS, sarcopenia, and anti-MuSK myasthenia gravis, the regimen for treatment usually stretches over many months or years, so oral dosing is preferred for patient convenience and tolerance. With oral dosing, one to five and especially two to four and typically three oral doses per day are representative regimens. Using these dosing patterns, each dose provides from about 0.01 to about 20 mg/kg of the compound of the invention, with preferred doses each providing from about 0.1 to about 10 mg/kg and especially about 1 to about 5 mg/kg. Long term treatment of the above-mentioned diseases is envisioned to span ~5 years with respect to ALS, ~20 years with respect to sarcopenia, and >30 years with respect to anti-MuSK myasthenia gravis.

Transdermal doses are generally selected to provide similar or lower blood levels than are achieved using injection doses. Modes of administration suitable for mucosal sites are also envisioned herein and include without limitation: intranasal swabs, enemas, intranasal sprays, and aerosolized or vaporized compounds and/or compositions for delivery to the lung mucosa. One of skill in the art would choose an appropriate delivery mode/s based on a variety of parameters, including the organ or tissue site in a patient with a disease or condition that is most severely affected by the disease or condition. A skilled practitioner could, for example, treat a patient afflicted with ALS, for example, with a therapeutic regimen that included delivery of the compounds or compositions of the invention using an intramuscular injection for direct delivery to an affected muscle. Intraperitoneal (ip) and intravenous (iv) injection delivery modes are also envisioned for the treatment of diseases and conditions described herein, including ALS.

When used to prevent the onset of a condition or disorder causally related or attributable to synaptic loss, the compounds of this invention will be administered to a patient at risk for developing the condition or disorder, typically on the advice and under the supervision of a physician, at the dosage levels described above. Patients at risk for developing a particular condition generally include those with a family history of the condition, or those who have been identified by genetic testing or screening to be particularly susceptible to developing the condition.

The compounds of this invention can be administered as the sole active agent or they can be administered in combination with other agents, including other compounds that demonstrate the same or a similar therapeutic activity and are determined to be safe and efficacious for such combined administration.

The invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention and should in no way be construed, however, as limiting the broad scope of the invention.

Experimental Procedures

5 Animals

Mice overexpressing human SOD1 (B6.Cg-Tg(SOD1-G93A)1Gur/J) were purchased from Jackson Laboratory (Bar Harbor, Me.) and crossed with HSA::MuSK-L transgenic mice, which were also maintained on a C57BL/6J background (24). SOD1G93A mice were genotyped by PCR (5'-CATCAGCCCTAATCCATCTGA-3' and 5'-CGCGACTAACAATCAAAGTGA-3'). Primers for IL-2 were used as an internal control (5'-CTAGGCCACAGAAATGAAAGATCT-3' and 5'-GTAGGTGGAAATTCTAGCATCATCC-3').

HSA::MuSK-L transgenic mice express 3-fold more MuSK than wild-type mice (Kim and Burden, 2008). Genotyping was performed by PCR (5'-GAAGCAACCTTTCCTTCCTGAG-3' and 5'-ATTTTCCTGAGAGCATTGTCC-3'). All experiments were approved by the Animal Care and Use Committee at NYU School of Medicine.

20 Immunohistochemistry

Diaphragm muscles from adult mice were dissected and fixed for 1.5 h at room temperature in 1% formaldehyde in phosphate buffered saline (PBS). Muscles were washed three times for 15 min in PBS, incubated for 15 min with 0.1M glycine in PBS and rinsed in PBS and 0.5% Triton X-100 (PBT). Muscles were incubated for 1 h in PBT containing 4% normal goat serum (PBTG), and overlaying connective tissue was diligently removed. Axons and nerve terminals were labeled with rabbit polyclonal antibodies against Neurofilament (NF, 1:3000; Synaptic Systems, Goettingen, Germany) and Synapsin (Syn, 1:2000; Synaptic Systems, Goettingen, Germany) overnight at 4° C. in PBTG. After three 1 h washes in PBT, muscles were incubated at 4° C. overnight with Alexa-488 goat anti-rabbit IgG (1:500; Invitrogen) and Alexa-594-conjugated- α -BGT (1:1000 in PBTG; Invitrogen, San Diego, Calif.) to label AChRs. Muscles were washed three times with PBS over 1 h, post fixed (1% formaldehyde in PBS) for 10 min, rinsed in PBS and Vectashield under a glass coverslip (Vector Labs, Burlingame, Calif.).

Diaphragm muscles from P90 to P160 wild-type (WT), HSA::MuSK-L, SOD1G93A and SOD1G93A; HSA::MuSK-L mice were stained with Alexa-594-conjugated- α -BGT and antibodies to NF and Syn. Confocal images of diaphragm muscles were captured on a Zeiss 510 confocal laser scanning microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) using a 40x PlanApo objective. Images were compiled into a reconstructed image, and the number of normally innervated, partially innervated and fully denervated synapses was quantified. For each experiment, at least 100 synaptic sites were counted for each genotype, and experiments were performed at least three times.

Survival

To measure longevity, we followed the survival of 300 animals up to 10 months: WT, n=101; HSA::MuSK-L, n=86; SOD1G93A, n=70; and SOD1G93A; HSA::MuSK-L, n=44. Kaplan Meier survival curves were generated with GraphPad Prism software.

60 Behavioral Tests

Motor function was assessed once per week on a Rota Rod (EZ-Rod 3.05; Accuscan Instruments, Inc., Columbus, Ohio). In each experiment, running performance was measured for at least 6 animals of each genotype from P80 to P160. Mice were placed on a Rota Rod (3.0 cm rotating cylinder) rotating at 1 rpm, and the speed of rotation was gradually increased from 1 to 12 rpm over the course of 40 sec and then main-

tained at 12 rpm for a maximum of five min. We recorded the time that mice remained on the Rota Rod. Wild-type and HSA::MuSK-L mice routinely ran for the full five min, yielding an assigned value of 100%; the values for other mice were expressed relative to wild-type mice.

Motor fatigue was assessed using an inverted grid hanging test (Kaja et al., 2007). Individual mice were placed in the center of a wire grid, which was mounted 80 cm above a laboratory bench. After gently inverting the grid, we maintained the grid in an inverted position for a maximum of 60 sec and recorded the time that mice remained attached to the grid. Wild-type mice routinely remained attached to the grid for the duration and were assigned a value of 100%; the values for other mice were expressed relative to wild-type mice.

Statistical Analysis

All data are expressed as group means \pm SEM. For the Kaplan-Meier survival analysis, the log-rank test was used, and survival curves were considered significantly different at $P<0.05$. When appropriate, the one-way ANOVA followed by a Newman-Keuls Multiple Comparison's post-hoc analysis were used to test for differences between samples, and data were considered significantly different at $P<0.05$.

hSOD1 Copy Number Assessment

To learn whether SOD1G93A copy number changed during the course of these experiments, copy number was measured using a real time PCR assay. Genomic DNA was extracted from tails using a Qiagen DNA extraction kit (QIAGEN, Valencia, Calif.). Brilliant® II SYBR Green QPCR Master Mix reagent (Stratagene, Santa Clara, Calif.) was used for the real-time amplification of DNA, ranging from 0.15 to 20 ng. Following heating at 50° C. for 2 min and 95° C. for 10 min, DNA was amplified by 40 cycles of 95° C. for 15 sec and 60° C. for 1 min., as suggested by the Jackson Laboratories (Bar Harbor, Me.). We found that the copy number did not change during the course of these experiments (FIG. 6).

Introduction

The withdrawal of motor axons from muscle is the first sign of disease in familial and sporadic forms of ALS, portending a progressive and devastating loss of motor function that culminates in lethal muscle paralysis within five years of diagnosis (Fischer et al., 2004; Pasinelli and Brown, 2006; Schaefer et al., 2005). The mechanisms responsible for axon withdrawal are poorly understood, but the loss of neuromuscular synapses is sufficient to cause muscle paralysis and therefore central to the disease. Although the subsequent loss of motor neurons has received more attention, preventing or delaying motor neuron cell death without preserving neuromuscular synapses cannot stop disease progression. Moreover, studies designed to inhibit motor neuron cell death, either by blocking cell death pathways or providing broadly acting growth factors, have had only a modest impact on disease progression (Kostic et al., 1997; Pun et al., 2006; Sagot et al., 1995).

Dominant mutations in SOD1, TDP-43, FUS, and the recently discovered C9orf72 gene are responsible for familial forms of ALS and together represent ~17% of all cases of ALS (Chen-Plotkin et al., 2010; Kwiatkowski et al., 2009; Majounie et al., 2012; Pasinelli and Brown, 2006; Renton et al., 2011; Vance et al., 2009). The pathological hallmarks of the disease are well replicated in mice that overexpress mutant forms of SOD1 in motor neurons, providing an excellent model system for studying the pathology of ALS and identifying approaches to treat this devastating disease (Pasinelli and Brown, 2006). Although cell types other than motor neurons, including grey matter oligodendrocytes and microglia contribute to disease onset and progression (Ilieva

et al., 2009; Kang et al., 2010), dominant mutations in SOD1 act largely in an autonomous manner within motor neurons, consistent with the idea that ALS is primarily, though not entirely, a disease of upper and lower motor neurons.

Skeletal muscles provide retrograde signals that promote the differentiation and stabilization of motor nerve terminals (Burden, 1998; Sanes and Lichtman, 2001). In the absence of these muscle-derived retrograde signals, developing motor axons grow aimlessly within muscle and fail to form synapses. The production of muscle-derived retrograde signals depends upon a synaptic receptor tyrosine kinase, termed MuSK, and Lrp4, a receptor for Agrin that forms a complex with MuSK (DeChiara et al., 1996; Kim et al., 2008; Weatherbee et al., 2006; Zhang et al., 2008). During normal development, Lrp4/MuSK signaling initiates neuromuscular synapse formation, whereas the subsequent stabilization of nascent synapses also requires neuronal Agrin, which binds to Lrp4 and strongly stimulates MuSK (Kim and Burden, 2008; Zhang et al., 2008; Zhang et al., 2011). In the absence of Agrin, neuromuscular synapses form, but motor axon terminals subsequently withdraw, leading to defective neuromuscular transmission and perinatal death (Gautam et al., 1996; Lin et al., 2001; Lin et al., 2005; Miggeld et al., 2005). Retrograde signaling also regulates the stability and maintenance of synapses in adult animals, as interfering with MuSK function in adult mice causes disassembly of neuromuscular synapses (Hesser et al., 2006; Kong et al., 2004). Because a failure to maintain neuromuscular synapses is central to all forms of ALS, we tested whether increasing retrograde signaling in SOD1 transgenic mice could stabilize neuromuscular synapses, delay axon withdrawal and ameliorate the symptoms of disease.

Results

Previously, we found that a modest (three-fold) increase in MuSK expression is sufficient to maintain neuromuscular synapses in agrin mutant mice, thereby preventing perinatal lethality and promoting postnatal survival (Kim and Burden, 2008). We therefore wondered whether increasing MuSK expression in a mouse model of ALS would stabilize neuromuscular synapses, delay motor axon withdrawal, and increase muscle function.

To investigate this issue, we crossed HSA::MuSK-L mice, which express three-fold more MuSK than wild-type mice, with SOD1G93A mice, and used histological assays to compare the rate and extent of denervation of SOD1G93A and MuSK-L; SOD1G93A mice. We stained whole mounts of the diaphragm muscle with antibodies against Synapsin to label nerve terminals and with α -bungarotoxin (α -BGT) to visualize acetylcholine receptors (AChRs) in muscle. In wild-type and MuSK-L transgenic mice, nerve terminals are apposed to AChRs, and the coincidence of Synapsin/AChR staining defines innervated synaptic sites (FIG. 1). In SOD1G93A mice, axons from fast, fatigable motor neurons withdraw early in disease (Pun et al., 2006). Axons from slow, non-fatigable motor neurons are lost more slowly and compensate by sprouting and temporarily reoccupying denervated synaptic sites on fast myofibers (Pun et al., 2006). We first measured the number of synaptic sites that lacked Synapsin staining and were therefore completely denervated. In SOD1G93A mice, denervation of the diaphragm muscle became evident at P120, and the extent of denervation increased gradually over the next 20 days, reaching a maximum of ~50% at P140 (FIG. 1), similar to the time course and extent of denervation observed in other muscles (Schaefer et al., 2005). In contrast, in SOD1G93A mice over-expressing MuSK, denervation of the diaphragm muscle began ten days later, and the extent of denervation remained less than 10% through P150 (FIG. 1).

By P160, the extent of denervation increased and approached the level found in SOD1G93A mice (FIGS. 1, 5). These findings indicate that MuSK over-expression protected synapses in SOD1G93A mice from denervation by delaying the onset and decreasing the extent of denervation for over 40

days. The remaining synapses included normally and partially innervated synapses. At partially innervated synapses, Synapsin staining incompletely overlapped the AChR-rich postsynaptic membrane, leaving patches of the postsynaptic membrane devoid of innervation (FIG. 2). To determine whether MuSK expression preserved normal innervation, we measured the number of synaptic sites that were normally innervated. FIG. 2 shows that the number of normally innervated synapses fell dramatically after P110 in SOD1G93A mice, reaching a plateau of 20% at P140. MuSK over-expression in SOD1G93A mice preserved normal innervation: the fall in normal innervation was delayed, and >60% of synapses remained normally innervated as late as P150. These findings show that MuSK over-expression preserves normally innervated synapses in SOD1G93A mice for over 40 days. MuSK over-expression may preserve innervation by delaying denervation, increasing reinnervation or both.

To determine whether the delay in onset and decrease in extent of denervation were accompanied by improved motor function, we used a Rota Rod and an inverted grid hanging test to measure the motor performance of SOD1G93A and MuSK-L; SOD1G93A mice. FIG. 3 shows that MuSK-L; SOD1G93A mice out-performed SOD1G93A mice in both tests of motor function. MuSK-L; SOD1G93A mice clung to the inverted grid longer than SOD1G93A mice, beginning at P120 and continuing through P160, the last time point that we measured. Although the motor function of MuSK-L; SOD1G93A mice declined over time, SOD1G93A mice over-expressing MuSK out-performed SOD1G93A mice by 2-3 fold at P120 and P130 and by 5-9 fold from P140 through P160 (FIG. 3a). Likewise, MuSK-L; SOD1G93A mice out-performed SOD1G93A mice on the Rota Rod from P100 through P160 (FIG. 3b). Moreover, these differences in motor behavior were evident simply by monitoring the mobility of mice in their housing. Nonetheless, the benefit from MuSK over-expression is not enduring, since the eventual withdrawal of nerve terminals and decrease in motor function leads to death at a time similar to SOD1G93A mice (FIG. 4a). Discussion

Results presented herein reveal that a modest increase in MuSK expression is sufficient to maintain neuromuscular synapses in SOD1G93A mice, delaying muscle denervation and improving muscle function for over 30 days. These findings indicate that the loss of motor nerve terminals can be delayed by co-opting a retrograde signaling pathway that normally functions to stimulate the differentiation and stabilization of these terminals. As such, our findings suggest a novel therapeutic approach to slow the steady decline in muscle strength and motor function in ALS. Moreover, because motor axon withdrawal is an early, characteristic and critical feature of disease in all forms of ALS, we expect that increasing MuSK activity might provide benefit in both familial and sporadic forms of ALS.

The benefit from MuSK over-expression is not permanent. The eventual withdrawal of nerve terminals and loss of motor function indicate that motor neurons ultimately become sufficiently compromised that motor terminals can no longer be stabilized by increasing MuSK signaling from muscle, suggesting that the terminals eventually lose their ability to respond to critical MuSK-dependent muscle-derived signals.

Our recent studies demonstrate that Lrp4 is a critical muscle-derived retrograde signal that acts bi-directionally to coordinate presynaptic and postsynaptic differentiation (Yumoto et al., Nature, DOI 10.1038/nature11348). A failure to transport the Lrp4 receptor, or components that act downstream from this receptor, within motor axons may ultimately render motor neurons unresponsive to retrograde signaling (FIG. 4b). Identification of the Lrp4 receptor might provide additional targets that can be manipulated to strengthen and prolong the response of compromised motor neurons. Alternatively, the eventual withdrawal of nerve terminals could be caused by the death of motor neurons, a late event in ALS that becomes apparent in SOD1G93A mice one to two months after denervation has reached a plateau (Fischer et al., 2004). If so, combining other therapies, aimed to promote motor neuron survival, with an increase in MuSK activity may lengthen the duration of benefit.

Like other receptor tyrosine kinases, tyrosine phosphorylation and activation of MuSK depend upon MuSK dimerization (Stiegler et al., 2009), which is stimulated by binding of Agrin to Lrp4 (Zhang et al., 2011). MuSK over-expression is sufficient to promote MuSK dimerization and increase MuSK kinase activity (Watty et al., 2000). It will be necessary, however, to find alternatives to MuSK over-expression to activate MuSK in vivo. Soluble forms of neuronal Agrin or Lrp4 may stimulate MuSK in vivo (Zhang et al., 2011), or a screen for small molecule activators of MuSK may identify new agonists. Human single chain variable region antibodies (ScFv) to MuSK have also been shown to stimulate MuSK tyrosine phosphorylation and AChR clustering in cultured myotubes (Xie et al., 1997). The mechanisms by which these agonist antibodies stimulate MuSK are poorly understood, but these antibodies may provide an alternative means to activate MuSK in vivo, an approach that we are currently investigating.

Example II

Further to the above findings, the present inventor is investigating whether proven agonist antibodies to MuSK stimulate MuSK phosphorylation in vivo and likewise improve motor function in ALS mice.

As indicated herein above, previous studies have shown that agonist antibodies to MuSK stimulate MuSK phosphorylation in cultured myotubes (Xie et al., 1997). To explore the properties of these agonist antibodies to MuSK within the context of the present disclosure, an exemplary one of these antibodies, agonist antibody #13 of Xie et al. (1997), will be evaluated. Xie et al. describe agonist antibody, #13, as the most potent of these antibodies. Accordingly, agonist antibody #13 will be assessed to determine if this antibody stimulates MuSK phosphorylation in mice. Because ScFv and truncated (Fab) antibodies have short (1-2 hr) half-lives in vivo, corresponding full length IgG molecules that are deficient in effector functions (e.g., complement activation) will also be assessed. Protocols for mutating IgG to ablate effector functions are known in the art as exemplified by Armour et al. (1999) and Shields et al. (2001), the entire content of each of which is incorporated herein in its entirety. Moreover, because the current version of antibody #13 includes a human constant region, the human heavy chain sequence contained in these antibodies will be exchanged with a murine heavy chain sequence to avoid generating an immune response in mice receiving injections for a month or longer. Using such protocols, the optimal dose, frequency and route of delivery for an agonist antibody can be determined. These experiments will establish a protocol for activating MuSK in vivo,

which information will provide additional guidance with respect to therapeutic applications of these agonist antibodies and the like.

More particularly, antibodies will be injected into wild-type mice at P100 and MuSK phosphorylation will be measured two, four or six days later. Additional time points may, moreover, be added. The antibodies will be injected into the peritoneum (IP) or intravenously (IV), and the dose will be varied from 0.1 mg/kg to 10 mg/kg. At the outset, IgG molecules will be injected that are defective in effector function, due to mutations in the lower hinge region that impair binding to Fcγ receptors (Armour et al., 1999; Shields et al., 2001), or by substituting the constant region from human IgG4 (or mouse IgG1), which inefficiently engages complement (Aalberse and Schuurman, 2002). These experiments will establish the optimal route for antibody delivery and the relationship between the amount of injected antibody and the level of MuSK phosphorylation. Moreover, these experiments will provide information as to the time required for the agonist antibody to activate MuSK following a single injection and the persistence of MuSK phosphorylation following a single injection.

MuSK phosphorylation can be measured, for example, by immunoprecipitating MuSK from cell lysates of dissected tibialis anterior, diaphragm or gastrocnemius muscles and probing Western blots for MuSK or tyrosine phosphorylation, as previously described (Friesse et al., 2007; Hallock et al., 2010; Herbst and Burden, 2000).

The above procedure can, furthermore, be modified by adding a second injection to evaluate whether the effects of repeated injections are predictably additive. It is unclear, for example, whether divalent, agonist IgG antibodies to MuSK down-regulate MuSK surface expression. Receptor down-regulation is likely to impact responsiveness to repeated injections. In one set of experiments, cultured myotubes will be treated with an agonist antibody and MuSK surface expression measured, as described previously (Stiegler et al. J Mol Biol 2006 364(3):424-433, the entire content of which is incorporated herein by reference). In addition, by injecting the agonist antibody a second time (or multiple times) in vivo, one or two weeks following the first injection, an assessment of whether the response to the agonist antibody has been attenuated, as would be expected if the first antibody treatment led to down regulation of MuSK surface expression, can be performed. Together, this knowledge will direct further experiments to establish how often injections will be required to achieve an adequate level of MuSK phosphorylation during the 40 to 80 day period required to determine whether the agonist antibody decreases muscle denervation and improves motor behavior, as described herein above.

Once optimal dosing, frequency and route of delivery for the agonist antibody are established, agonist antibody or a control antibody will be injected into SOD1G93A mice, beginning at P100, prior to the onset of muscle denervation and motor dysfunction, and injections will be continued through P140, when muscle denervation and motor dysfunction have reached peak levels. During this time frame, histological and behavioral assays can be performed as described herein above to make a determination as to whether the agonist antibody delays the onset and extent of denervation, as well as improves motor behavior in SOD1G93A mice.

To determine whether the agonist antibody slows disease progression when injected after the onset of disease symptoms, antibody injections can be commenced at P120, after muscle denervation and motor dysfunction in SOD1G93A mice are well apparent and at ~one-half their peak values. As shown herein above, the extent of muscle denervation reaches

~one-half the peak value by P120 and motor dysfunction has likewise deteriorated to a similar extent by this age. See, for example, FIG. 1. In view of the above, agonist antibody injections will commence at P120 and muscle denervation and motor dysfunction will be assessed at P140. These experiments will reveal the ability of an agonist antibody tested to slow, halt or reverse disease progression.

The prolonged impact of an agonist antibody on disease, reducing denervation, improving motor function and prolonging survival beyond P140 can, moreover, be assessed by continuing antibody injections from P140 to P180. As described above, a modest increase in MuSK expression delayed the onset and decreased the extent of muscle denervation, improving motor behavior up to P150. By P160, however, denervation was similar in SOD1G93A mice over-expressing MuSK and SOD1G93A mice, and survival was not significantly prolonged. Nonetheless, it is possible that activating MuSK with the agonist antibody may prevent denervation for a longer period of time and prolong survival. To explore this potentiality, the ability of agonist antibody to alter the extent of denervation at P180 and prolong survival will also be assessed by continuing injections from P140 to P180.

Upon establishing that an agonist antibody improves motor function in ALS mice, assessments of these antibodies will be performed in a clinical setting to determine therapeutic efficacy of the antibodies in human subjects.

In proof of principle experiments, cDNAs encoding three antibodies to MuSK were re-synthesized, two of which were reported by Xie et al. (1997) to function as agonist antibodies, whereas the third was determined to bind to MuSK, but failed to stimulate MuSK. These three antibodies, as well as an additional negative control antibody (all of which were synthesized as IgG molecules) were tested to determine if they recognize mouse and human MuSK using an ELISA. These antibodies were also tested to assess their ability to stimulate MuSK phosphorylation when added to cultured mouse myotubes. As shown in FIG. 7, the agonist antibodies do indeed stimulate robust MuSK phosphorylation. The negative control antibody, as well as another antibody that binds MuSK, failed to stimulate MuSK phosphorylation. Given these findings, the properties of two agonist antibodies, #13 and #22, to MuSK have been confirmed in vitro and have thus set the stage for the above-outlined in vivo experiments. The in vitro studies also revealed that the agonist antibodies react with mouse and human MuSK, although there is a slight preference for human MuSK.

This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all aspects illustrative and not restrictive, the scope of the invention being indicated by the appended claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

Various references are cited throughout this Specification, each of which is incorporated herein by reference in its entirety.

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SEQUENCE LISTING

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<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 2

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Gly Thr Val Glu Glu Ile Leu Asn Val Asp Pro Val Gln His Thr Tyr
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Ser Cys Lys Val Arg Val Trp Arg Tyr Leu Lys Gly Lys Asp Leu Val
 65           70           75           80
Ala Arg Glu Ser Leu Leu Asp Gly Gly Asn Lys Val Val Ile Ser Gly
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Phe Gly Asp Pro Leu Ile Cys Asp Asn Gln Val Ser Thr Gly Asp Thr
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Arg Ile Phe Phe Val Asn Pro Ala Pro Pro Tyr Leu Trp Pro Ala His
115          120          125
Lys Asn Glu Leu Met Leu Asn Ser Ser Leu Met Arg Ile Thr Leu Arg
130          135          140
Asn Leu Glu Glu Val Glu Phe Cys Val Glu Asp Lys Pro Gly Thr His
145          150          155          160
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Cys Gly Phe Gly Ala Val Cys Glu Pro Asn Ala Glu Gly Pro Gly Arg
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	545	550 555 560
Leu His Pro Met Glu Gly Thr Ile Tyr Trp Thr Asp Trp Gly Asn Thr		
	565	570 575
Pro Arg Ile Glu Ala Ser Ser Met Asp Gly Ser Gly Arg Arg Ile Ile		
	580	585 590
Ala Asp Thr His Leu Phe Trp Pro Asn Gly Leu Thr Ile Asp Tyr Ala		
	595	600 605
Gly Arg Arg Met Tyr Trp Val Asp Ala Lys His His Val Ile Glu Arg		
	610	615 620
Ala Asn Leu Asp Gly Ser His Arg Lys Ala Val Ile Ser Gln Gly Leu		
	625	630 635 640
Pro His Pro Phe Ala Ile Thr Val Phe Glu Asp Ser Leu Tyr Trp Thr		
	645	650 655
Asp Trp His Thr Lys Ser Ile Asn Ser Ala Asn Lys Phe Thr Gly Lys		
	660	665 670
Asn Gln Glu Ile Ile Arg Asn Lys Leu His Phe Pro Met Asp Ile His		
	675	680 685
Thr Leu His Pro Gln Arg Gln Pro Ala Gly Lys Asn Arg Cys Gly Asp		
	690	695 700
Asn Asn Gly Gly Cys Thr His Leu Cys Leu Pro Ser Gly Gln Asn Tyr		
	705	710 715 720
Thr Cys Ala Cys Pro Thr Gly Phe Arg Lys Ile Ser Ser His Ala Cys		
	725	730 735
Ala Gln Ser Leu Asp Lys Phe Leu Leu Phe Ala Arg Arg Met Asp Ile		
	740	745 750
Arg Arg Ile Ser Phe Asp Thr Glu Asp Leu Ser Asp Asp Val Ile Pro		
	755	760 765
Leu Ala Asp Val Arg Ser Ala Val Ala Leu Asp Trp Asp Ser Arg Asp		
	770	775 780
Asp His Val Tyr Trp Thr Asp Val Ser Thr Asp Thr Ile Ser Arg Ala		
	785	790 795 800

Lys	Trp	Asp	Gly	Thr	Gly	Gln	Glu	Val	Val	Val	Asp	Thr	Ser	Leu	Glu
				805					810						
Ser	Pro	Ala	Gly	Leu	Ala	Ile	Asp	Trp	Val	Thr	Asn	Lys	Leu	Tyr	Trp
				820					825						
Thr	Asp	Ala	Gly	Thr	Asp	Arg	Ile	Glu	Val	Ala	Asn	Thr	Asp	Gly	Ser
				835					840						
Met	Arg	Thr	Val	Leu	Ile	Trp	Glu	Asn	Leu	Asp	Arg	Pro	Arg	Asp	Ile
				850					855						
Val	Val	Glu	Pro	Met	Gly	Gly	Tyr	Met	Tyr	Trp	Thr	Asp	Trp	Gly	Ala
				865					870						
Ser	Pro	Lys	Ile	Glu	Arg	Ala	Gly	Met	Asp	Ala	Ser	Gly	Arg	Gln	Val
				885					890						
Ile	Ile	Ser	Ser	Asn	Leu	Thr	Trp	Pro	Asn	Gly	Leu	Ala	Ile	Asp	Tyr
				900					905						
Gly	Ser	Gln	Arg	Leu	Tyr	Trp	Ala	Asp	Ala	Gly	Met	Lys	Thr	Ile	Glu
				915					920						
Phe	Ala	Gly	Leu	Asp	Gly	Ser	Lys	Arg	Lys	Val	Leu	Ile	Gly	Ser	Gln
				930					935						
Leu	Pro	His	Pro	Phe	Gly	Leu	Thr	Leu	Tyr	Gly	Glu	Arg	Ile	Tyr	Trp
				945					950						
Thr	Asp	Trp	Gln	Thr	Lys	Ser	Ile	Gln	Ser	Ala	Asp	Arg	Leu	Thr	Gly
				965					970						
Leu	Asp	Arg	Glu	Thr	Leu	Gln	Glu	Asn	Leu	Glu	Asn	Leu	Met	Asp	Ile
				980					985						
His	Val	Phe	His	Arg	Arg	Arg	Pro	Pro	Val	Ser	Thr	Pro	Cys	Ala	Met
				995					1000						
Glu	Asn	Gly	Gly	Cys	Ser	His	Leu	Cys	Leu	Arg	Ser	Pro	Asn	Pro	Ser
				1010					1015						
Gly	Phe	Ser	Cys	Thr	Cys	Pro	Thr	Gly	Ile	Asn	Leu	Leu	Ser	Asp	Gly
				1025					1030						
Lys	Thr	Cys	Ser	Pro	Gly	Met	Asn	Ser	Phe	Leu	Ile	Phe	Ala	Arg	Arg
				1045					1050						
Ile	Asp	Ile	Arg	Met	Val	Ser	Leu	Asp	Ile	Pro	Tyr	Phe	Ala	Asp	Val
				1060					1065						
Val	Val	Pro	Ile	Asn	Ile	Thr	Met	Lys	Asn	Thr	Ile	Ala	Ile	Gly	Val
				1075					1080						
Asp	Pro	Gln	Glu	Gly	Lys	Val	Tyr	Trp	Ser	Asp	Ser	Thr	Leu	His	Arg
				1090					1095						
Ile	Ser	Arg	Ala	Asn	Leu	Asp	Gly	Ser	Gln	His	Glu	Asp	Ile	Ile	Thr
				1105					1110						
Thr	Gly	Leu	Gln	Thr	Thr	Asp	Gly	Leu	Ala	Val	Asp	Ala	Ile	Gly	Arg
				1125					1130						
Lys	Val	Tyr	Trp	Thr	Asp	Thr	Gly	Thr	Asn	Arg	Ile	Glu	Val	Gly	Asn
				1140					1145						
Leu	Asp	Gly	Ser	Met	Arg	Lys	Val	Leu	Val	Trp	Gln	Asn	Leu	Asp	Ser
				1155					1160						
Pro	Arg	Ala	Ile	Val	Leu	Tyr	His	Glu	Met	Gly	Phe	Met	Tyr	Trp	Thr
				1170					1175						
Asp	Trp	Gly	Glu	Asn	Ala	Lys	Leu	Glu	Arg	Ser	Gly	Met	Asp	Gly	Ser
				1185					1190						
Asp	Arg	Ala	Val	Leu	Ile	Asn	Asn	Asn	Leu	Gly	Trp	Pro	Asn	Gly	Leu
				1205					1210						

Thr	Val	Asp	Lys	Ala	Ser	Ser	Gln	Leu	Leu	Trp	Ala	Asp	Ala	His	Thr
			1220					1225					1230		
Glu	Arg	Ile	Glu	Ala	Ala	Asp	Leu	Asn	Gly	Ala	Asn	Arg	His	Thr	Leu
		1235					1240					1245			
Val	Ser	Pro	Val	Gln	His	Pro	Tyr	Gly	Leu	Thr	Leu	Leu	Asp	Ser	Tyr
		1250				1255					1260				
Ile	Tyr	Trp	Thr	Asp	Trp	Gln	Thr	Arg	Ser	Ile	His	Arg	Ala	Asp	Lys
1265					1270					1275					1280
Gly	Thr	Gly	Ser	Asn	Val	Ile	Leu	Val	Arg	Ser	Asn	Leu	Pro	Gly	Leu
				1285					1290					1295	
Met	Asp	Met	Gln	Ala	Val	Asp	Arg	Ala	Gln	Pro	Leu	Gly	Phe	Asn	Lys
			1300					1305					1310		
Cys	Gly	Ser	Arg	Asn	Gly	Gly	Cys	Ser	His	Leu	Cys	Leu	Pro	Arg	Pro
		1315				1320					1325				
Ser	Gly	Phe	Ser	Cys	Ala	Cys	Pro	Thr	Gly	Ile	Gln	Leu	Lys	Gly	Asp
	1330					1335					1340				
Gly	Lys	Thr	Cys	Asp	Pro	Ser	Pro	Glu	Thr	Tyr	Leu	Leu	Phe	Ser	Ser
1345					1350					1355					1360
Arg	Gly	Ser	Ile	Arg	Arg	Ile	Ser	Leu	Asp	Thr	Ser	Asp	His	Thr	Asp
				1365					1370					1375	
Val	His	Val	Pro	Val	Pro	Glu	Leu	Asn	Asn	Val	Ile	Ser	Leu	Asp	Tyr
			1380					1385					1390		
Asp	Ser	Val	Asp	Gly	Lys	Val	Tyr	Tyr	Thr	Asp	Val	Phe	Leu	Asp	Val
		1395				1400						1405			
Ile	Arg	Arg	Ala	Asp	Leu	Asn	Gly	Ser	Asn	Met	Glu	Thr	Val	Ile	Gly
	1410					1415					1420				
Arg	Gly	Leu	Lys	Thr	Thr	Asp	Gly	Leu	Ala	Val	Asp	Trp	Val	Ala	Arg
1425					1430					1435					1440
Asn	Leu	Tyr	Trp	Thr	Asp	Thr	Gly	Arg	Asn	Thr	Ile	Glu	Ala	Ser	Arg
				1445				1450						1455	
Leu	Asp	Gly	Ser	Cys	Arg	Lys	Val	Leu	Ile	Asn	Asn	Ser	Leu	Asp	Glu
			1460					1465					1470		
Pro	Arg	Ala	Ile	Ala	Val	Phe	Pro	Arg	Lys	Gly	Tyr	Leu	Phe	Trp	Thr
		1475				1480						1485			
Asp	Trp	Gly	His	Ile	Ala	Lys	Ile	Glu	Arg	Ala	Asn	Leu	Asp	Gly	Ser
	1490					1495					1500				
Glu	Arg	Lys	Val	Leu	Ile	Asn	Thr	Asp	Leu	Gly	Trp	Pro	Asn	Gly	Leu
1505					1510					1515					1520
Thr	Leu	Asp	Tyr	Asp	Thr	Arg	Arg	Ile	Tyr	Trp	Val	Asp	Ala	His	Leu
				1525					1530					1535	
Asp	Arg	Ile	Glu	Ser	Ala	Asp	Leu	Asn	Gly	Lys	Leu	Arg	Gln	Val	Leu
			1540					1545					1550		
Val	Ser	His	Val	Ser	His	Pro	Phe	Ala	Leu	Thr	Gln	Gln	Asp	Arg	Trp

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1635	1640	1645
Ser Leu Val Pro Gly Leu Val Pro Pro Ala Pro Arg Ala Thr Gly Met		
1650	1655	1660
Ser Glu Lys Ser Pro Val Leu Pro Asn Thr Pro Pro Thr Thr Leu Tyr		
1665	1670	1675 1680
Ser Ser Thr Thr Arg Thr Arg Thr Ser Leu Glu Glu Val Glu Gly Arg		
	1685	1690 1695
Cys Ser Glu Arg Asp Ala Arg Leu Gly Leu Cys Ala Arg Ser Asn Asp		
	1700	1705 1710
Ala Val Pro Ala Ala Pro Gly Glu Gly Leu His Ile Ser Tyr Ala Ile		
	1715	1720 1725
Gly Gly Leu Leu Ser Ile Leu Leu Ile Leu Val Val Ile Ala Ala Leu		
	1730	1735 1740
Met Leu Tyr Arg His Lys Lys Ser Lys Phe Thr Asp Pro Gly Met Gly		
1745	1750	1755 1760
Asn Leu Thr Tyr Ser Asn Pro Ser Tyr Arg Thr Ser Thr Gln Glu Val		
	1765	1770 1775
Lys Ile Glu Ala Ile Pro Lys Pro Ala Met Tyr Asn Gln Leu Cys Tyr		
	1780	1785 1790
Lys Lys Glu Gly Gly Pro Asp His Asn Tyr Thr Lys Glu Lys Ile Lys		
	1795	1800 1805
Ile Val Glu Gly Ile Cys Leu Leu Ser Gly Asp Asp Ala Glu Trp Asp		
	1810	1815 1820
Asp Leu Lys Gln Leu Arg Ser Ser Arg Gly Gly Leu Leu Arg Asp His		
1825	1830	1835 1840
Val Cys Met Lys Thr Asp Thr Val Ser Ile Gln Ala Ser Ser Gly Ser		
	1845	1850 1855
Leu Asp Asp Thr Glu Thr Glu Gln Leu Leu Gln Glu Glu Gln Ser Glu		
	1860	1865 1870
Cys Ser Ser Val His Thr Ala Ala Thr Pro Glu Arg Arg Gly Ser Leu		
	1875	1880 1885
Pro Asp Thr Gly Trp Lys His Glu Arg Lys Leu Ser Ser Glu Ser Gln		
	1890	1895 1900
Val		
1905		

What is claimed is:

1. A method for delaying motor dysfunction in a subject suffering from amyotrophic lateral sclerosis (ALS), the method comprising the steps of: selecting the subject suffering from ALS and administering an agent that increases muscle specific receptor kinase (MuSK) activity to the subject in a therapeutically effective amount sufficient to increase MuSK activity in the subject, wherein the agent is an antibody or antibody fragment that increases MuSK activity upon binding to MuSK, thereby improving motor function in the subject.

2. A method for preserving neuromuscular synapses in a subject suffering from amyotrophic lateral sclerosis (ALS), the method comprising the steps of: selecting the subject suffering from ALS and administering an agent that increases muscle specific receptor kinase (MuSK) activity to the subject in a therapeutically effective amount sufficient to increase MuSK activity in the subject, wherein the agent is an antibody

or antibody fragment that increases MuSK activity upon binding to MuSK, thereby preserving neuromuscular synapses in the subject.

3. The method of claim 1, wherein the delay in motor dysfunction is measurable by determining innervation levels.

4. The method of claim 1, wherein administering the agent maintains innervation levels in the subject.

5. The method of claim 1, wherein administering the agent stabilizes motor axon synapses or increases the number of motor axon synapses in the subject.

6. The method of claim 1, wherein the subject is a mouse comprising a transgene with a mutation in superoxide dismutase (SOD1).

7. The method of claim 6, wherein the mouse comprises a transgene with a mutation in SOD1, wherein the transgene is SOD1G93A.

8. The method of claim 1, wherein the subject is a human.

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